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(54) HEAT TREATMENT TO PRODUCE GLYCOSIDES

WÄRMEBEHANDLUNG ZUR HERSTELLUNG VON GLYKOSIDEN

TRAITEMENT THERMIQUE POUR PRODUIRE DES GLYCOSIDES

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(56) References cited:
WO-A1-2013/110673
WO-A2-2014/191581
US-A1- 2006 083 838
US-A1- 2006 083 838
US-A1- 2012 164 678
US-A1- 2014 329 281
US-A1- 2015 031 868
US-A1- 2015 037 462

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Description

Background

[0001] Sugars, such as sucrose, fructose and glucose, are utilized to provide a pleasant taste to beverages, foods, pharmaceuticals, oral hygienic and cosmetic products. Sucrose, in particular, imparts a taste preferred by most consumers. Although sucrose provides superior sweetness characteristics, it is caloric. Non-caloric or lower caloric sweeteners have been introduced to satisfy consumer demand. Consumers also desire that these sweeteners have favorable taste characteristics.

[0002] Stevia is a genus of about 240 species of herbs and shrubs in the sunflower family (*Asteraceae*), native to subtropical and tropical regions and from western North America to South America. The species *Stevia rebaudiana,* commonly known as sweetleaf, sweet leaf, sugarleaf, or simply stevia, is widely grown for its sweet leaves. Stevia-based sweeteners may be obtained by extracting one or more sweet compounds from the leaves. Many of these compounds are glycosides of steviol, a diterpene compound. These diterpene glycosides are about 150 to 450 times sweeter than sugar. Steviol glycosides differ from each other by sweetness power as well as other sensory features contributing to taste quality such as bitterness, lingering aftertaste and the like. See Kinghorn, A. D., Stevia: The genus Stevia, Taylor & Francis, London (2002).

[0003] Examples of steviol glycosides are described in PCT International Patent Application Publication No. WO 2013/096420 (see, e.g., listing in Fig. 1); and in Ohta et. al., "Characterization of Novel Steviol Glycosides from Leaves of Stevia rebaudiana Morita," J. Appl. Glycosi., 57, 199-209 (2010) (see, e.g., Table 4 at p. 204). Structurally, the diterpene glycosides are characterized by a single base, steviol, and differ by the presence of carbohydrate residues at positions C13 and C19, as presented in Figs. 2a-2k of WO 20013/096420.

[0004] Typically, on a dry weight basis, the four major steviol glycosides found in the leaves of Stevia are dulcoside A (0.3%), rebaudioside C (0.6-1.0%), rebaudioside A (3.8%) and stevioside (9.1%). Other glycosides identified in Stevia extract include one or more of rebaudioside B, D, E, F, G, H, I, J, K, L, M, N, O, steviolbioside and rubusoside.

[0005] While the major steviol glycoside, rebaudioside A, is commonly used as sweetener in beverage applications it has off-taste issues. More recently, there has been focus on certain minor steviol glycosides which have better taste properties. For example, rebaudioside M has higher sweetness intensity and is more potent than other steviol glycosides (e.g., see Prakash, I., et al. (2013) Nat. Prod. Commun., 8: 1523-1526, and WO 2013/096420). Rebaudioside D tastes about 200-220 times sweeter than sucrose and in a sensory evaluation had a slow onset of sweetness and was very clean, namely sweeter overall than sucrose, less sweet lingering
aftertaste compared to sucrose (e.g., see Prakash, I., et al. (2012) Int. J. Mol. Sci., 13:15126-15136).

[0006] Recombinant DNA technology has made it possible, and commercially viable, to produce desired steviol glycosides using a wide variety of host cells such as yeast. Recovery of intracellularly expressed products from within the host cells, however, is fraught with difficulties because of the necessity of rupturing or hydrolyzing the tough yeast cell wall. The choice of cell wall disruption can also affect the downstream processing of the recovered product and its subsequent purification from yeast cells or fermentation broths thereby affecting the efficiency and cost effectiveness of the process.

[0007] WO2013110673A describes a recombinant microorganism capable of producing diterpene and the preparation of the diterpene comprising fermenting the recombinant microorganism. WO2013110673A discloses that broth was incubated at 95° C for 5 minutes, acetonitrile was added, samples were mixed, spun down and diluted for analyzing the steviol or steviol glycoside by using LC/MS.

[0008] WO2014191581A describes recombinant microorganism for diterpene production. WO2014191581A discloses the cultures in the 96-well plate was incubated for 15 minutes at 95° C and cooled down, and to each well acetonitrile was added and homogenized for analyzing Reb A using LC/MS.

[0009] US2014329281A describes recombinant microorganisms that have been engineered to express recombinant genes encoding USTs and can produce steviol glycosides. US2014329281A discloses that DMSO was added to the culture samples which were vortexed, heated at 80° C for 15 minutes, and the steviol glycosides or 19-SMG content was analyzed by LC-MS.

Summary

[0010] Disclosed is a method to efficiently recover desired steviol glycosides produced by recombinant yeast technology in a cost effective manner.

[0011] The invention discloses a method of releasing steviol glycosides from a yeast host cell, comprising heating a fermentation medium comprising steviol glycoside producing engineered yeast host cells to a temperature in the range from 70° C to 75° C for 5 minutes to 48 hours to release one or more steviol glycosides from the engineered host cells into the fermentation medium, wherein the steviol glycoside is selected from rebaudioside M, rebaudioside D, rebaudi-

oside A, rebaudioside B or combinations thereof.

[0012] The invention discloses a method of releasing steviol glycosides from a yeast host cell, comprising the steps of:

a) providing a fermentation medium comprising a yeast host cell engineered to produce one or more steviol glycosides; and
b) releasing the one or more steviol glycosides from the engineered yeast host cell by heating the composition to a temperature in the range from 70° C to 75° C for 5 minutes to 48 hours,
wherein the one or more steviol glycosides are released from an intracellular portion of the host cell into an extracellular portion of the fermentation medium, wherein the steviol glycoside is selected from rebaudioside M, rebaudioside D, rebaudioside A, rebaudioside B or combinations thereof.

[0013] A method of releasing steviol glycosides from a composition, the method comprising the step of heating the composition to a temperature in the range from 50° C to 95° C for 5 minutes to 48 hours to release the steviol glycosides from engineered host cells in the composition is described.

[0014] A method of releasing steviol glycosides, the method comprising the steps of:

a) providing a composition comprising an engineered host cell to produce one or more steviol glycosides; and
b) releasing the one or more steviol glycosides from the engineered host cell by heating the composition to a temperature in the range from 70° C to 75° C for 5 minutes to 48 hours is described.

[0015] A composition produced by the method according to the invention, comprising an engineered yeast host cell to produce one or more steviol glycosides, and one or more steviol glycosides that are released from the engineered host cell by heating the composition to a temperature in the range from 70° C to 75° C for 5 minutes to 48 hours is described.

[0016] Such methods result in releasing the glycosides into the composition while minimizing cell lysis.

Description of the Drawings

[0017]

FIG. 1 shows a representative mevalonate pathway.
FIG. 2 shows a representative non-mevalonate pathway.
FIG. 3 shows a representative pathway for steviol production.
FIG. 4 shows representative pathways for the biosynthesis of steviol glycosides from steviol.
FIG. 5A is a digital image of a bright field microscopy image before heat treatment.
FIG. 5B is a digital image of a bright field microscopy image after heat treatment.

Detailed Description

[0018] Embodiments of the disclosure described herein are not intended to be exhaustive or to limit the invention to the precise forms disclosed in the following detailed description. Rather a purpose of the embodiments chosen and described is the appreciation and understanding by others skilled in the art of the claims. The publications and patents disclosed here are provided solely for their disclosure.

[0019] Host cells such as yeast and fungus can be engineered for the synthesis of steviol glycosides. During fermentation only a fraction of the glycosides produced by the host cell may be released from within the cells into the fermentation media. To increase the glycoside amount released from the host cell such as yeast, mechanical and chemical disruption methods may be used. Mechanical disruption, namely the breaking of the cell, releases the intracellular components into the surrounding medium. The result is a complex mixture of not only the desired recombinant product but also other products such as protein, nucleic acids, cellular metabolites, cell fragments, complicating the recovery of the desired product. Chemical methods entail chemically altering or making permeable the cell membrane and/or wall structure to allow diffusion of the product from within the cell. Chemical treatments, however, have to be compatible with the organism and the release of the desired product.

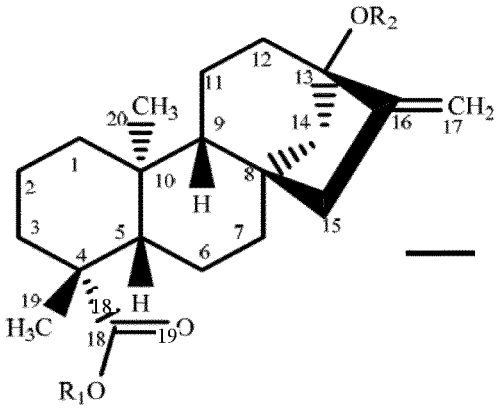
[0020] Described are methods of efficiently recovering steviol glycosides after fermentation using heat treatments. Heat applied for a given time period and temperature to a fermentation broth or fermentation medium that includes host cells (e.g. yeast and fungus). The host cells can produce the desired steviol glycosides. When the fermentation medium containing the host cells are heated, the desired glycosides can be released from the host cells with minimal lysis of the cells thereby enriching the fermentation media or composition for the desired glycoside.

[0021] The method of the invention uses engineered yeast capable of producing steviol glycosides. An engineered yeast capable of producing steviol glycosides can include one or more exogenous nucleic acids that encode enzyme(s)

that promote formation of one or more steviol glycosides in the cell.

[0022] As used herein, the term "steviol glycoside(s)" refers to glycosides of steviol. Exemplary steviol glycoside, include, but are not limited to, rebaudioside A, rebaudioside B, rebaudioside C, rebaudioside D, rebaudioside E, rebaudioside F, rebaudioside G, rebaudioside H, rebaudioside I, rebaudioside J, rebaudioside K, rebaudioside L, rebaudioside M, rebaudioside N, rebaudioside O, stevioside, steviolbioside, dulcoside A, and/or rubusoside. Engineered yeast can produce steviol glycosides that are naturally found ("naturally occurring") or ones that are not found in nature (non-naturally occurring). As used herein, the term "total steviol glycosides" (TSG) is calculated as the sum of the content of all steviol glycosides in a composition on a dry (anydrous) basis.

[0023] Structurally, steviol glycosides have a central molecular moiety, which is a single steviol base, and glucopyranosyl residues, or other sugar residuals attached to the C_{13} and C_{19} atoms of the steviol base, according to the atom numbering on the base shown below. That is, glucopyranosyl residues represent groups R_1 and R_2 in the following formula:



[0024] Table A below shows the certain steviol glycosides and the corresponding R_1 and R_2 groups:

Table A

Compound name	R_1 (C-19)	R_2 (C-13)
Steviol	H	H
Stevioside	β-Glu	β-Glu-β-Glu (2->1)
Rebaudioside A	β-Glu	β-Glu-β-Glu (2->1) | β-Glu (3->1)
Rebaudioside B	H	β-Glu-β-Glu (2->1) | β-Glu (3->1)
Rebaudioside C	β-Glu	β-Glu-α-Rha (2->1) | β-Glu (3->1)
Rebaudioside D	β-Glu-β-Glu (2->1)	β-Glu-β-Glu (2->1) |
Rebaudioside E	β-Glu-β-Glu (2->1)	β-Glu-β-Glu (2->1)
Rebaudioside G	β-Glu	β-Glu-β-Glu (3->1)

(continued)

Compound name	R_1 (C-19)	R_2 (C-13)
Rebaudioside M	β-Glu-β-Glu (2->1) | β-Glu (3->1)	β-Glu-β-Glu (2->1) | β-Glu (3->1)
Rebaudioside N	β-Glu-α-Rha (2->1) | β-Glu (3->1)	β-Glu-β-Glu (2->1) | β-Glu (3->1)
Rebaudioside O	β-Glu-α-Rha (2->1)- β-Glu (3->1) | β-Glu (3->1)	β-Glu-β-Glu (2->1) | β-Glu (3->1)
Glu: glucose Rha: rhamnose		

[0025] The steviol glycosides may be produced in a fermentation process The fermentation process can use a genetically modified organism that is engineered for the production of one or more steviol glycosides, such as rebaudioside A (Reb A), rebaudioside M (Reb M) and/ or rebaudioside D (Reb D). Production of steviol glycosides can be carried out using an engineered microbial strain having a set of enzymes that provide a pathway for the synthesis of steviol glycosides.

[0026] Various yeast or fungal host cells can be engineered to provide a pathway to one or more steviol glycosides. Such cells can be transformed with one or more DNA construct encoding enzymes for steviol glycoside synthesis. Exemplary yeast and fungus that can be used for hosts for exogenous DNA constructs encoding steviol glycoside pathway enzymes, include, but are not limited to the genus of, *Candida, Pichia* (*Hansenula*), *Kloeckera* (*Hanseniaspora*), *Kluyveromyces, Rhodotorula, Torulopsis, Zygosaccharomyces, Saccharomycete, Yarrowia,* and *Saccharomyces.* Exemplary species include *Candida albicans, Pichia pastoris, Saccharomyces cerevisiae,* and *Schizosaccharomyces pombe.* Further, host cells can also include genetic modifications other than those of the steviol glycoside pathway that may provide improved performance during fermentation.

[0027] An "engineered host cell" refers to yeast and fungal cells having at least one exogenous DNA sequence that is introduced into the cell, either integrated into the cell's genome or present on an extrachromosomal construct, such as a plasmid in bacteria or episomes. While the specification may describe yeast cells in detail, it should be understood that the method may also be adapted to fungal cells.

[0028] The term "exogenous" refers to a molecule, such as a nucleic acid, or an activity, such as an enzyme activity. The exogenous molecule is introduced into the host yeast or fungus. An exogenous nucleic acid can be introduced in to the host organism by well-known techniques and can be maintained external to the hosts chromosomal material (e.g., maintained on a non-integrating vector), or can be integrated into the host's chromosome, such as by a recombination event. Generally, the genome of an engineered yeast or fungus is augmented through the stable introduction of one or more recombinant genes. An exogenous nucleic acid can encode an enzyme, or portion thereof, that is either homologous or heterologous to the host organism. An exogenous nucleic acid can be in the form of a "recombinant gene or DNA construct" referring to a nucleic acid that is in one or more ways manipulated through molecular techniques to be in a form that does not naturally exist. The term "non-natural" may be used to characterize a molecule, such as a nucleic acid or protein, or an organism that does not naturally exist in nature.

[0029] The term "heterologous" (e.g., "non-native") refers to a molecule or activity that is from a source that is different than the referenced molecule or organism. Accordingly, a gene or protein that is heterologous to a referenced organism is a gene or protein not found in that organism. In the context of the disclosure, a "heterologous glycosyltransferase" refers to a glycosyltransferase polypeptide that is different from any glycosyltransferase polypeptide that may be native to the host organism. For example, a specific glycosyltransferase gene found in a first species and exogenously introduced into a host yeast or fungal organism that is different than the first species is "heterologous" to the host yeast or fungal organism.

[0030] The engineered yeast or fungus can use an auxotrophic marker suitable for selecting for a transformant. The host cell can include modifications (e.g. deletions) in one or more genes that control auxotrophies, such as LYS2, LEU2, HIS3, URA3, URA5, and TRP1. Using a host cell having a desired genetic background for introduction of one or more exogenous genes, one or more gene construct(s) is introduced into a cell to integrate into the genome, or to be stably maintained and allow for expression. Methods for introducing a gene construct into a host cell include transformation,

transduction, transfection, co-transfection, electroporation. In particular, yeast transformation can be carried out using the lithium acetate method, the protoplast method, and the like. The gene construct to be introduced may be incorporated into a chromosome in the form of a plasmid, or by insertion into the gene of a host, or through homologous recombination with the gene of a host. The transformed yeast into which the gene construct has been introduced can be selected with a selectable marker (for example, an auxotrophic marker as mentioned above). Further confirmation can be made by measuring the activity of the expressed protein.

[0031] The transformation of exogenous nucleic acid sequences including the steviol pathway genes can be confirmed using methods well known in the art. Such methods include, for example, nucleic acid analysis such as Northern blots or polymerase chain reaction (PCR) amplification, or immunoblotting for expression of gene products, or other suitable analytical methods to test the expression of the introduced nucleic acid sequences or their corresponding gene product. It is understood by those skilled in the art that the exogenous nucleic acid is expressed in an amount sufficient to produce the desired product, and it is further understood that expression levels can be optimized to obtain sufficient expression using methods well known in the art and as disclosed herein.

[0032] The terpenoid compounds, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), can serve as chemical precursors to steviol glycosides in an engineered host cell (e.g. yeast). Some organisms, including plants, insect, and some microbial species, have a mevalonate (MVA) pathway that converts acetyl-CoA through a series of chemical intermediates to IPP and DMAPP. Some organisms produce IPP and DMAPP through the non-mevalonate pathway (also known as the methyl D-erythritol 4-phosphate or MEP pathway) starting with glyceraldehyde-3-phosphate (G3P) and pyruvate (PYR).

[0033] The yeast *Saccharomyces cerevisiae* naturally expresses genes of the mevalonate pathway. Mevalonate pathway genes include: (a1) acetoacetyl CoA thiolase (EC 2.3.1.9), (b1) 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) synthase (EC 4.1.3.5); (c1) HMG-CoA reductase (EC 1.1.1.34); (d1) mevalonate kinase (EC 2.7.1.36); (e1) phosphomevalonate kinase (EC 2.7.4.2); and (f1) mevalonate diphosphate decarboxylase (EC 4.1.1.33). Mevalonate pathway enzymes convert acetyl-CoA to IPP as follows: acetyl-CoA → acetoacetyl-CoA → 3-hydroxy-3-methylglutaryl-CoA → mevalonate → mevalonate-5-phosphate → mevalonate-5-pyrophosphate → IPP. See also FIG. 1

[0034] The engineered yeast can include one or more modifications to increase the flux from acetyl-CoA to IPP and/or DMAPP, thereby providing an increased pool of IPP and/or DMAPP for use in a pathway to steviol. The modifications can include, for example, increasing expression or activity of one or more mevalonate pathway enzymes (a1)-(f1) , such as by placing a nucleic acid encoding an enzyme that is homologous or heterologous to the yeast cell under the control of a promoter that provides increased expression, using multiple copies of the nucleic acid, and/or using a heterologous enzyme, a variant enzyme (e.g., including one or more amino acid substitutions), or a variant heterologous enzyme that provides a higher level of enzymatic activity as compared to the native enzyme.

[0035] Alternatively, the non-mevalonate (MEP) pathway can be used to provide IPP and DMAPP as precursors to steviol glycoside production. The yeast *Saccharomyces cerevisiae* does not naturally express genes of the MEP pathway, but can optionally be engineered to provide MEP pathway genes. Theoretically, the MEP pathway is more energetically efficient generally because it loses less carbon as CO2 as compared to the MVA pathway (MEP pathway: 1 CO2/IPP; MVA pathway: 4 CO2/IPP; sugar as carbon source).

[0036] In particular, the non-mevalonate (MEP) pathway compounds, isopentenyl diphosphate (IPP), dimethylallyl diphosphate (DMAPP), are generated through a series of intermediates leading from glyceraldehydes-3-phosphate (G3P) and pyruvate (PYR), and a number of enzymes are responsible for this conversion. Enzymes involved in a biosynthetic pathway from G3P and PYR to IPP and DMAPP include (a2) 1-deoxy-D-xylulose-5-phosphate synthase (DXS), (b2) 1-Deoxy-D-xylulose-5-phosphate reductoisomerase (ispC)-, (c2) 4-diphosphocytidyl-2C-methyl- D-erythritol synthase (IspD), (d2) 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (IspE), (e2) 2C- Methyl-D-erythritol-2,4-cyclodiphosphate Synthase (IspF), (f2) 1-hydroxy-2-methyl-2-(E)-butenyl-4- diphosphate synthase (IspG), (g2) 4-hydroxy-3-methyl-2-(E)-butenyl-4-diphosphate reductase (IspH), and (h2) isopentenyl-diphosphate isomerase (IDI). See FIG. 2

[0037] The engineered yeast used to produce steviol glycosides by fermentation can have one or more genetic modifications to increase the flux from G3P and PYR to IPP and/or DMAPP, thereby providing an increased pool of IPP and/or DMAPP for use in a pathway to steviol. The modifications can include, for example, increasing expression or activity of one or more enzymes (a2) - (h2), such as by placing a nucleic acid encoding an enzyme that is heterologous to the yeast cell under the control of a promoter that provides increased expression, using multiple copies of the nucleic acid, and/or using a heterologous enzyme, a variant enzyme (e.g., one including one or more amino acid substitutions), or a variant heterologous enzyme that provides a high levels of enzymatic activity.

[0038] The engineered yeast used to produce steviol glycosides by fermentation can also include a pathway to convert IPP and/or DMAPP to steviol. For example, in some aspects the engineered yeast can include exogenous nucleic acids expressing the following enzymes: (a3) geranyl geranyldiphosphate synthase (GGPPS), (b3) copalyl diphosphate synthase (CPS), (c3) kaurene synthase (KS), (d3) kaurene oxidase (KO), and (e3) kaurenoic acid 13- hydroxylase (KAH). Enzymes of the mevalonate pathway converts IPP and/or DMAPP to steviol as follows: IPP/ DMAPP → geranyl geranyldiphosphate → copalyl diphosphate → kaurene → kaurenoic acid → steviol. (See FIG. 3) Exogenous nucleic acids

encoding enzymes (a3) - (e3) that are heterologous to the yeast cell can be placed under the control of a promoter that provides increased expression, using multiple copies of the nucleic acid, and/or using a variant enzyme (e.g., one including one or more amino acid substitutions), or a variant heterologous enzyme that provides a high levels of enzymatic activity.

[0039] The methods of the disclosure for producing steviol glycoside(s) by fermentation can use engineered yeast including one or more steviol biosynthesis enzymes selected from the group of geranylgeranyl diphosphate synthase, copalyl diphosphate synthase, kaurene synthase, kaurene oxidase, kaurenoic acid 13-hydroxylase (KAH), steviol synthetase, deoxyxylulose 5-phosphate synthase (DXS), D-1-deoxyxylulose 5-phosphate reductoisomerase (DXR), 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase (CMS), 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (CMK), 4-diphosphocytidyl-2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MCS), 1-hydroxy-2-methyl-2(E)-butenyl 4-diphosphate synthase (HDS), 1-hydroxy-2-methyl-2(E)-butenyl 4-diphosphate reductase (HDR), acetoacetyl-CoA thiolase, truncated HMG-CoA reductase, mevalonate kinase, phosphomevalonate kinase, mevalonate pyrophosphate decarboxylase, cytochrome P450 reductase etc.

[0040] The engineered yeast used to produce steviol glycosides by fermentation can have any pathway to convert steviol to a steviol glycoside. If more than one steviol glycoside pathway enzymes are present in the engineered yeast, the yeast may be able to produce different steviol glycosides. For example, the yeast may be able to produce two, three, four, five, six, seven, eight, nine, ten, or more than 10 different steviol glycoside species. Reb A, Reb B, Reb D and/or Reb M are produced by the engineered yeast cells of the method of the invention.

[0041] The steviol glycoside pathway can include one or more uridine diphosphate (UDP) glycosyltransferases (UGTs) that mediate the transfer of glycosyl residues from activated nucleotide sugars to acceptor molecules. In the case of a steviol glycoside pathway, a monosaccharide unit can be transferred to a hydroxyl or carboxyl moiety on a steviol or steviol glycoside molecule, or to a hydroxyl group on a glucose group that is attached to the steviol base. See FIG. 4. UGTs have been classified into families and subfamilies based on sequence homology. See Li et al., 2001, J. Biol. Chem. 276:4338-4343. A superfamily of over 100 genes encoding UGTs, each containing a 42 amino acid consensus sequence, has been identified in the model plant *Arabidopsis thaliana,* and genes encoding UGTs have also been identified in several other higher plant species.

[0042] Exemplary UDP-glucosyltransferase can be any UDP-glucosyltransferase capable of adding at least one glucose unit to the steviol and or steviol glycoside substrate to provide the desired steviol glycoside. The engineered yeast can include one or more UDP-glucosyltransferase selected from group UGT74G1 (SEQ ID NO: 1), UGT85C2 (SEQ ID NO: 2), UGT76G1 (SEQ ID NO: 3), UGT91D2 (SEQ ID NO: 4), and also UGTs having substantial (e.g. >85%, >75%, >65%, >55%, >45% and >35%) identity to these polypeptides. An engineered yeast can include one or more exogenous nucleic acid molecule(s) that code for these UGTs.

[0043] The engineered yeast can also include one or more UGT and UDP-glucose recycling enzyme(s). An exemplary UDP-glucosyltransferase capable of adding at least one glucose unit to rubusoside to form stevioside is UGT91D2 (SEQ ID NO: 4). An exemplary UDP-glucosyltransferase capable of adding at least one glucose unit to stevioside to form rebaudioside A is UGT76G1 (SEQ ID NO: 3). An exemplary UDP-glucosyltransferase capable of adding at least one glucose unit to rebaudioside A to form rebaudioside D is UGT91D2 (SEQ ID NO: 4). An exemplary UDP-glucosyltransferase capable of adding at least one glucose unit to rebaudioside D to form rebaudioside M is UGT76G1 (SEQ ID NO: 3).

[0044] Exemplary publications that describe engineered microorganisms for steviol glycoside production include, for example, U.S. Patent Application Publication No. 2014/0357588, International Patent Application Publication Nos. WO 2014/193934, WO 2014/193888, and WO 2014/122227.

[0045] An engineered yeast useful for the production of steviol glycosides may express the following enzymes: geranylgeranyl diphosphate synthase (GGPPS), ent-copalyl diphosphate synthase (CDPS), kaurene oxidase (KO), kaurene synthase (KS); steviol synthase (KAH), cytochrome P450 reductase (CPR), UGT74G1, UGT76G1, UGT91D2, UGT85C2 and a EUGT11. WO2014/122227 describes an engineered yeast strain that express these enzymes. The UDP-glucosyltransferases can be a gene encoding a polypeptide for example, UGT74G1 (SEQ ID NO: 1), UGT85C2 (SEQ ID NO: 2), UGT76G1 (SEQ ID NO: 3), UGT91D2 (SEQ ID NO: 4), and a EUGT11 (SEQ ID NO: 13); these genes encode polypeptides capable of carrying out a number of reactions such as a) a gene encoding a polypeptide capable of beta 1,2 glucosylation of the C2' of the 19-*O* glucose of a steviol glycoside; (b) a gene encoding a polypeptide capable of beta 1,2 glucosylation of the C2' of the 13-*O*-glucose of a steviol glycoside; (c) a gene encoding a polypeptide capable of beta 1,3 glucosylation of the C3' of the 19-*O*-glucose of a steviol glycoside; (d) a gene encoding a polypeptide capable of beta 1,3 glucosylation of the C3' of the 13-*O*-glucose of a steviol glycoside; (i) a gene encoding a polypeptide capable of glucosylation of the 13-OH of steviol or a steviol glycoside;(j) a gene encoding a polypeptide capable of glucosylation of the C-19 carboxyl of steviol or a steviol glycoside. For example, UGT85C2 carries out reaction (i); UGT74G1 carries out reaction (j); UGT91D2 carries out reactions (a; weakly), (b); UGT76G1 carries out reactions (c) and (d) EUGT11 carries out reactions (a), (b; less well).

[0046] The engineered host cell may express one or more exogenous nucleic acid(s) encoding one or more of the following proteins heterologous to the host cell: GGPPS polypeptide, an enf-copalyl diphosphate synthase (CDPS)

polypeptide, a kaurene oxidase (KO) polypeptide, a kaurene synthase (KS) polypeptide; a steviol synthase (KAH) polypeptide, a cytochrome P450 reductase (CPR) polypeptide, a UGT74G1 polypeptide, a UGT76G1 polypeptide, a UGT91 d2 polypeptide, and a EUGT11 polypeptide.

[0047] In another embodiment, the engineered yeast expresses one or more exogenous nucleic acid(s) encoding one or more of the following proteins heterologous to the host cell: a GGPPS polypeptide, a truncated *Zea mays* CDPS polypeptide, an *Arabidopsis thaliana* KS polypeptide a *Stevia rebaudiana* KO polypeptide, an *Arabidopsis thaliana* ATR2 polypeptide, an *Oryzya sativa* EUGT 11 polypeptide, a SrKAHel polypeptide, a *Stevia rebaudiana* CPR8 polypeptide, an *Stevia rebaudiana* UGT85C2 polypeptide, an *Stevia rebaudiana* UGT74G1 polypeptide, a *Stevia rebaudiana* UGT76G1 polypeptide, a *Stevia rebaudiana* UGT91D2 variant or functional homolog, and a UGT91D2e-b polypeptide.

[0048] Steviol glycoside-producing *S. cerevisiae* strains were constructed using methods as described in WO 2011/153378, WO 2013/022989, WO 2014/122227, and WO 2014/122328. The following sequences were used for construction of a parent strain (Strain A): a recombinant gene encoding a *Synechococcus* sp GGPPS polypeptide (SEQ ID NO:6), a recombinant gene encoding a truncated *Zea mays* CDPS polypeptide (SEQ ID NO:7), a recombinant gene encoding an *Arabidopsis thaliana* KS polypeptide (SEQ ID NO:8), a recombinant gene encoding a recombinant *Stevia rebaudiana* KO polypeptide (SEQ ID NO:9, SEQ ID NO:10), a recombinant gene encoding an *Arabidopsis thaliana* ATR2 polypeptide (SEQ ID NO:11, SEQ ID NO:12), a recombinant gene encoding an *Oryza sativa* EUGT 11 polypeptide (SEQ ID NO:13), a recombinant gene encoding an SrKAHel polypeptide (SEQ ID NO:14, SEQ ID NO:15), a recombinant gene encoding an *Stevia rebaudiana* CPR8 polypeptide (SEQ ID NO:16, SEQ ID NO:17), a recombinant gene encoding an *Stevia rebaudiana* UGT85C2 polypeptide (SEQ ID NO:2), a recombinant gene encoding an *Stevia rebaudiana* UGT74G1 polypeptide (SEQ ID NO:1), a recombinant gene encoding an *Stevia rebaudiana* UGT76G1 polypeptide (SEQ ID NO:3), and a recombinant gene encoding an *Stevia rebaudiana* UGT91D2 variant (or functional homolog), UGT91D2e-b, (SEQ ID NO:4) polypeptide produced steviol glycosides.

[0049] The UGT91D2e-b variant of UGT91D2 (SEQ ID NO:5 from PCT/US2012/050021) includes a substitution of a methionine for leucine at position 211 and a substitution of an alanine for valine at position 286. (Additional variants, except T144S, M152L, L213F, S364P, and G384C variants, described in Table 12 and Example 11 of PCT/US2012/050021 could be used.) GeneArt codon-optimized sequence encoding a *Stevia rebaudiana* UGT91D2e-b with the amino acid modifications L211M and V286A (SEQ ID NO:4 for amino acid sequence; codon optimized nucleotide sequence is set forth in SEQ ID NO:5).

[0050] Strain B is derived from the parent strain described above and additionally includes a codon-optimized CPR1 from *Stevia rebaudiana* (SEQ ID NO: 18 corresponding to amino acid SEQ ID NO: 19).

[0051] The engineered yeast may express one or more exogenous nucleic acid(s) encoding one or more of the following proteins heterologous to the yeast: GGPPS polypeptide, an ent-copalyl diphosphate synthase (CDPS) polypeptide, a kaurene oxidase (KO) polypeptide, a kaurene synthase (KS) polypeptide; a steviol synthase (KAH) polypeptide, a cytochrome P450 reductase (CPR) polypeptide, a UGT74G1 polypeptide, a UGT76G1 polypeptide, a UGT91D2 polypeptide, and a EUGT11 polypeptide.

[0052] The engineered yeast may express one or more exogenous nucleic acid(s) encoding one or more of the following proteins heterologous to the yeast: a GGPPS polypeptide, a truncated *Zea mays* CDPS polypeptide, an *A. thaliana* KS polypeptide a *S. rebaudiana* KO polypeptide, an *A. thaliana* ATR2 polypeptide, an *O. sativa* EUGT 11 polypeptide, a SrKAHel polypeptide, a *S. rebaudiana* CPR8 polypeptide, an *S. rebaudiana* UGT85C2 polypeptide, an *S. rebaudiana* UGT74G1 polypeptide, a *S. rebaudiana* UGT76G1 polypeptide, a *S. rebaudiana* UGT91D2 variant or functional homolog, and a UGT91D2e-b polypeptide

[0053] The engineered host cells (e.g., yeast cells) can be used to produce the desired steviol glycosides such as rebaudioside M, rebaudioside D, and rebaudioside A by fermentation. "Fermentation" as used here refers to the assimilation of the medium such as the carbohydrates to produce the desired steviol glycosides through aerobic fermentation. The term "medium" or grammatical equivalents such as "media" refers to a liquid composition in which the engineered yeast or fungus can be maintained, grown, or fermented, or combinations thereof. A "medium" may also be referred to as a "broth" or "cell culture," and terms such as "fermentation" that qualifies the term "medium" and its equivalents may be used to more specifically define the type of cellular activity that is occurring in the medium.

[0054] A medium can be defined with regards to the components present in the medium, and amounts thereof, including, but not limited to: (a) carbon sources, including carbohydrates such as glucose and starch products such as maltodextrin; (b) nitrogen sources, such as yeast nitrogen base, ammonium hydroxide, urea, ammonium sulfate, yeast extract or any combination thereof; (c) salts, such as potassium phosphate (monobasic, dibasic), magnesium sulfate, sodium chloride, and calcium chloride; (d) vitamins, such as biotin, calcium pantothenate, folic acid, (myo)-inositol, nicotinic acid, p-aminobenzoic acid, pyridoxine HCl, riboflavin, thiamine HCL, and citric acid; and/or (e) trace metals such as boric acid, copper sulfate, cobalt chloride, calcium chloride, potassium iodide, ferric chloride, magnesium sulfate, manganese chloride, sodium molybdate, and zinc sulfate. The medium can also be defined with regards to its pH, and biocompatible acids, bases, and buffers that are used to control the pH in the medium.

[0055] Fermentation of the engineered yeast can be performed using starch and/or sugar containing plant material

derivable from any plant and plant part, such as tubers, roots, stems, leaves and seeds. Starch and/or sugar comprising plant material can be obtained from cereal, such as barley, wheat, maize, rye, sorghum, millet, barley, potatoes, cassava, or rice, and any combination thereof. The starch-and/or sugar comprising plant material can be processed, such as by methods such as milling, malting, or partially malting. The fermentation media may include a treated starch. For example, the fermentation media can include a partially hydrolyzed starch. The partially hydrolyzed starch can include high molecular weight dextrins and high molecular weight maltodextrins. A partially hydrolyzed starch product can be used that have amounts of starch and starch degradation products within desired ranges.

[0056] Fermentation can be carried out under conditions and in medium suitable for production of the desired steviol glycosides such as rebaudioside M, rebaudioside D, and rebaudioside A. The fermentation conditions generally use oxygen (aerobic conditions), a carbon source, and a nutrient (nitrogen) base. Fermentation can be carried out using a fed batch or continuous process. The fermentation minimal medium includes glucose (5g/L), ammonium sulfate (5g/L), potassium dihydrogenphosphate (3g/L), magnesium sulphate (0.5 g/L), trace elements, and vitamins (e.g., see, Verduyn, C. et al. (1992) Yeast 8, 501-517). The pH of the fermentation media can be at about pH 4 to pH 5 and the fermentation can be carried out at a temperature at about 30° C. Fermentation can be carried out using a first growth phase in base medium, followed by a longer feeding phase using a glucose-containing defined feed medium (with trace metals, vitamins, and salts).

[0057] Fermentation may be carried out using a pH greater than 5.0. The pH may range from about 5.5 to 8 or 5.8 to 7.5. The engineered yeast may be grown initially at a lower pH such as less than 6.0 and fermentation carried out a higher pH such as greater than 5.0. Other methods of fermentation for producing the desired steviol glycosides are described in the application titled "Fermentation Methods for Producing Steviol Glycosides Using High pH and Compositions Obtained Therefrom," U.S. Pat. No. 10815513, and in International PCT application titled "Fermentation Methods for Producing Steviol Glycosides," PCT/US2016/034728 and filed concurrently with the present application.

[0058] Optionally, fermentation can be carried out in media containing other intermediates such as steviol, rebudioside A, stevioside, rubusoside, steviol monoside, steviolbioside, dulcoside A, rebaudioside C, rebaudioside E, and the like.

[0059] The fermentation step can also be referred to as a "steviol glycoside producing step." The fermentation medium may be maintained at a temperature less than 40° C during the fermentation step. The fermentation medium may be maintained at a temperature in the range of about 25 to 35° C during the fermentation step.

[0060] After fermentation, but before any treatment (e.g., heat treatment) to release or enrich for the desired steviol glycosides, the fermentation broth may contain only a fraction of the desired glycosides, with the majority of the desired glycosides within the engineered host cell. Disclosed are methods of releasing or enriching for the desired glycoside from the yeast host cell by using heat.

[0061] The heat treatment may be carried out on the whole cell fermentation broth, which contains the engineered yeast capable of producing the desired steviol glycosides and the desired steviol glycoside products. The engineered yeast after fermentation but before any treatment (e.g. heat treatment) may be separated from the fermentation broth and transferred into any suitable composition such as a liquid media.

[0062] The fermentation broth or composition containing the transferred yeast cells is heated at temperatures ranging from 70°C to 75°C for 5 minutes to 48 hours. Heating may be 70°C to 75°C for 5 minutes to 2 hours, or 75°C for 5 minutes to 2 hours. The heating may be at 70°C to 75°C for 5 minutes or from 70°C to 75°C for 2 hours.

[0063] The heat treatment releases the steviol glycosides into the medium or enriches the steviol glycoside content of the medium such that the extracellular glycoside content is greater than 90% of the total (i.e., both intracellular and extracellular) glycoside content in the fermentation broth or composition. The heat treatment step may release steviol glycosides into the fermentation medium such that the extracellular steviol glycoside content is greater than 80%, 85%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, of the total (i.e., both intracellular and extracellular) steviol glycoside content in the fermentation broth or The heat treatment step may release steviol glycosides into the fermentation medium such that the extracellular steviol glycoside content is in the range of about 80 to 100%, 85 to 100%, 90 to 100%, 90 to 99%, 92 to 98%, or 90 to 95% of the total (i.e., both intracellular and extracellular) steviol glycoside content in the fermentation broth or composition.

[0064] The heat treatment of the fermentation broth or composition releases Reb A, Reb B, Reb M, Reb D or combinations thereof. In some embodiments, the heat treatment enriches for Reb A such that the extracellular Reb A is greater than 50 % of the total (i.e., both intracellular and extracellular) Reb A present in the fermentation broth or composition. In some embodiments, the extracellular Reb A is greater than 40 %, 45%, 48%, 52%, 55%, 57%, 60%, 62%, 65%, 67%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% of the total (i.e., both intracellular and extracellular) Reb A present in the fermentation broth or composition. In some embodiments, the Reb A amount, can be released extracellularly in the range of 30% to 100 %, 50 % to 100 %, 80 % to 90 % of the total amount of Reb A produced during the fermentation.

[0065] In some embodiments, the heat treatment enriches for Reb D and/or Reb M such that the Reb D and/or Reb M released extracellularly is greater than 33 % of the total (i.e. both intracellular and extracellular) Reb D and/or Reb M present in the fermentation broth or composition. In some embodiments, the extracellular Reb D, Reb M, and/or the total of Reb D and Reb M is greater than 40 %, 45%, 48%, 52%, 55%, 57%, 60%, 62%, 65%, 67%, 70%, 75%, 80%, 85%,

90%, 95%, or 99% of the total (i.e., both intracellular and extracellular) Reb D, Reb M, and/or the total of Reb D and Reb M present in the fermentation broth or composition. In some embodiments, the combined amount of Reb D and Reb M, released extracellularly can be present in the range of 30% to 100%, 50% to 100%, 80% to 90% of the total amount of Reb D and/or Reb M present in the fermentation broth or composition.

[0066] In other embodiments, the Reb D released, can be present extracellularly in the range of 30% to 100%, 50% to 100%, 80% to 90 % of the total amount of Reb D present in the fermentation broth or composition.

[0067] In other embodiments, the Reb M released, can be present extracellularly in the range of 30% to 100%, 50% to 100%, 80% to 90% of the total amount of Reb M present in the fermentation broth or composition.

[0068] In some embodiments, the Reb B released, can be present extracellularly in the range of 25% to 100%, 30% to 95%, 40% to 80% of the total amount of Reb B present in the fermentation broth or composition. In some embodiments, the extracellular Reb B is greater than 30%. 33%. 35%, 40 %, 45%, 48%, 52%, 55%, 57%, 60%, 62%, 65%, 67%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% of the total (i.e., both intracellular and extracellular) Reb B present in the fermentation broth or composition. The disclosed heat treatment may not result in significant degradation from Reb DM and/or Reb A to Reb B.

[0069] The disclosed heat treatment may result in the release of the desired glycosides yet may not result in extensive lysis of the cell. Cell lysis may be determined, for example, by the amount of nitrogen or phosphorus or both released into the fermentation broth (e.g., cell free) or composition after the fermentation and heat treatment. The heating of the fermentation broth or composition may not increase nitrogen and/or phosphorus content in the cell free fermentation broth or composition. The extracellular nitrogen content in the cell free fermentation broth or composition after the heat treatment may not increase by more than 45% of the total (i.e. extracellular and intracellular) nitrogen present in the whole cell fermentation broth or composition. The extracellular phosphorous content in the fermentation broth or composition after the heat treatment may not increase by more than 70% of the total (i.e. extracellular and intracellular) phosphorous present in the cell-containing fermentation broth or composition.

[0070] The extracellular nitrogen content may not increase by more than 45% of the total nitrogen present in the cell-containing fermentation broth or composition and/or the extracellular phosphorous content in the fermentation broth or composition after the heat treatment may not increase by more than 70% of the total phosphorous present in the cell-containing fermentation broth or composition when heated at 70°C to 75°C for 5 minutes to 2 hours.

[0071] When the fermentation medium is heated at 70°C to 75°C for 5 minutes to 48 hours, the extracellular nitrogen content may not increase by more than 30%, 33%, 35%, 37%, 40%, 42%, 47%, or 50% of the total nitrogen present in the cell-containing fermentation broth or When the fermentation medium is heated at 70°C to 75°C for 5 minutes to 48 hours, the extracellular phosphorus content may not increase by more than 50%, 55%, 60%, 63%, 65%, 67%, 73%, 75%, 77%, or 80% of the total phosphorus present in the cell-containing fermentation broth.

[0072] The intracellular and/or extracellular content of various components of the fermentation broth (e.g., steviol glycosides, nitrogen, or phosphorus) can be determined as described in Example 1.

[0073] The heat treatment of the fermentation broth may also be evaluated based on the cell viability. While the desired glycosides may be released from the yeast cell into the fermentation broth, the yeast viability is decreased while limiting cell lysis. Cell lysis may be determined, for example, by plating the whole cell broth on Potato Dextrose Antibiotic Agar or by cell viability counter (e.g., Cellometer™).

[0074] Heating may be carried out by using a heat exchanger in which heat is transferred to hot water or steam. Heating may be carried out using a heat exchanger with a holding loop, direct steam injection, or bulk heating of the material utilizing steam applied to a jacketed vessel. The yeast may be concentrated for example, by centrifugation or filtration, before a heat treatment. The centrifuged or filtered yeast may be transferred into other suitable media or composition and then used for heating.

[0075] Following the release of the desired recombinant product (e.g. steviol glycosides) from the host cell into the medium, thereby enriching for the desired product, various procedures can be used for further isolation and purification of the desired recombinant product. Such procedures include centrifugation, ultrafiltration, precipitation and chromatographic procedures.

[0076] After the steviol glycosides are released or enriched, the steviol glycosides may be further purified by preparative HPLC as described, for example in International Patent Application Publication No. WO 2009/140394.

[0077] The fermentation broth or composition after the heat treatment can then be centrifuged or filtered to remove the engineered cells. The fermentation broth can optionally be treated to remove low molecular weight components (glucose, basic nutrients, and salts), such as by membrane dialysis.

[0078] If it is desired to provide a composition with steviol glycosides including Reb A, Reb B, Reb M, Reb D, and combinations thereof in enriched or purified form, or where Reb A, Reb B, Reb M, Reb D, and combinations thereof are separated from other steviol glycosides, or separated from one another, further purification can be carried out. Such enrichment or purification of steviol glycoside components can be carried out on liquid fermentation media, or the fermentation media can then be dried down prior to purification. For example, fermentation media can be dried down using lyophilization to form a dry composition (e.g., powder or flakes) including steviol glycosides with Reb A, Reb B,

Reb M, Reb D, and combinations thereof that can be subsequently processed.

[0079] Dried fermentation broth or composition enriched for steviol glyosides including Reb A, Reb B, Reb M, and/or Reb D, may be used as the starting material for purification. For example, a solvent or solvent combination can be added to the dried fermentation broth to dissolve or suspend materials that include the steviol glycosides. An exemplary combination for dissolving the steviol glycosides is a mixture of water and an alcohol (e.g., 50:50 ethanol:water). To facilitate dissolving or resuspending, the dried broth materials can be heated at a temperature above room temperature, such as in the range of 40°C - 60°C. Mechanical disruption of the dried fermentation broth materials can also be performed, such as by sonication. The dissolved or resuspended fermentation broth materials can be filtered using a micron or submicron filtration system prior to further purification, such as by preparative chromatography.

[0080] Dried fermentation broth or composition enriched for steviol glycoside compounds can be subjected to other purification processes, such as reverse phase liquid chromatography. A suitable resin can be used to retain steviol glycoside compounds in the column, with removal of hydrophilic compounds which get washed through the column with a liquid such as water. Elution of steviol glycosides including Reb A, Reb B, Reb M, Reb D, and combinations thereof from the column can be accomplished using a suitable solvent or solvent combination such as acetonitrile or methanol.

[0081] The steviol glycosides such as Reb A, Reb B, Reb M, Reb D, and combinations thereof can be purified using preparative liquid chromatography, such as high pressure liquid chromatography (HPLC) or ultra high pressure liquid chromatography (UHPLC). A steviol glycoside composition with Reb A, Reb B, Reb M, Reb D, and combinations thereof can be dissolved in a mobile phase, such as a mixture of water and an solvent (e.g., methanol, ethanol, acetonitrile) at a desired ratio (e.g., 60% water, 40% methanol, v/v). The composition can also be heated to enhance dissolution of the steviol glycoside material, such as heating at about 50°C. The solution can also be filtered prior to injection into the column, such as using a 0.2 μm filter. Phenomenex Kinetex XB-C18 5 μm, core-shell silica solid support, and stationary phase of C18 with iso-butyl side chains and TMS endcapping. The flow rate through the column can be based on column properties (such as about 20 mL/min), with a maximum pressure of 400 bar. Reb A, Reb B, Reb M, Reb D, and combinations thereof can be identified by their elution times from the column. In exemplary flow conditions Reb A, Reb B, Reb M, Reb D, and combinations thereof can elute from the column within 60 minutes. One of skill in the art will appreciate that the elution times for the Reb A, Reb B, Reb M, Reb D, and combinations thereof can vary with changes in solvent and/or equipment. One of skill in the art will also understand that although the process described below assumes certain order of the described steps, this order can be altered in some cases.

[0082] The fermentation broth or composition enriched for steviol glyosides including Reb A, Reb M, and/or Reb D, may be dried and used as the starting material for further purification. A steviol glycoside composition with Reb A, Reb M, Reb D, and combinations thereof can be dissolved in water and passed through adsorption chromatographic column to remove hydrophilic impurities in the composition. Adsorbed steviol glycosides can be desorbed from the column with ethanol/water mixture. Upon removal of the ethanol, the steviol glycoside liquid stream can be further purified with ion exchange chromatography to remove ions and color bodies. Additional treatment with activated carbon could be performed. To further separate specific steviol glycosides from others, a crystallization step could be used. The crystals may be enriched with Reb M, Reb D, Reb A, or other steviol glycosides. The crystals can be dried under heat and vacuum or can be re-slurried with water and spray dried.

Example 1

Production Of Reb D, Reb M And Reb A In Fed Batch Fermentation With Ammonium Hydroxide As The Primary Nitrogen Source

[0083] For inoculum preparation, a yeast strain designated strain B was cultured in 150 mLs of seed flask medium in 1 liter shake flasks at 250 rpm and 30°C for 20-24 hours.

Table 1 - Seed Flask Medium

Component	Formula	Concentration	Units
Biospringer 0251 yeast extract		7.5	g/L
Glucose monohydrate	$C_6H_{12}O_6 * H_2O$	22.0	g/L

[0084] For the fermentation, 75 mL of seed culture was transferred into initial fermentation medium (Tables 2, 3 and 4) with a starting volume of 0.75 liters. Temperature was maintained at 30°C throughout. The air flow rate was 1.75 SLPM and the agitation rate was automatically controlled to increase in a stepwise manner from 400 to 900 rpm during the fermentation. Glucose concentration was kept limiting by controlling flow rates of feed medium (Table 5). A 2-phase

feeding strategy involved an initial exponential phase beginning at 10 hours with a growth rate of u= 0.12 1/h while the second phase of feeding (or feed phase II) started at 33 hours with a constant flow rate of 0.180 mls/minute. Feeding was continued until a final volume of 1.95 liters was obtained by 120 hours.

[0085] pH was maintained at pH 5 with 12% NH_4OH throughout fermentation. Antifoam addition was controlled by utilization of foam control probes with 10 wt% antifoam solution (Ivanhoe 1163B). The medium was based on Verduyn et al (Verduyn C, Postma E, Scheffers WA, Van Dijken JP. Yeast. 1992 Jul; 8(7):501-17) with modifications as described in Tables 2 through 5.

Table 2 - Initial Fermentation Medium

Component	Formula	Concentration	Units
Glucose monohydrate	$C_6H_{12}O_6$* H_2O	22.0	g/L
Ammonium sulfate	$(NH_4)_2SO_4$	5.0	g/L
Monobasic potassium phosphate	KH_2PO_4	3.0	g/L
Magnesium sulfate heptahydrate	$MgSO_4$* 7 H_2O	0.5	g/L
Trace metals stock		10.0	ml/L
Vitamin stock		12.0	ml/L

Table 3 - Trace Metals Stock Solution

Component	Formula	Concentration	Units
Disodium edetate	$C_{10}H_{14}N_2Na_2O_8$ * $2H_2O$	15	g/L
Zinc sulfate heptahydrate	$ZnSO_4$ * $7H_2O$	4.5	g/L
Manganese (II) chloride tetrahydrate	$MnCl_2$ * $4H_2O$	1.026	g/L
Cobalt (II) chloride hexahydrate	$CoCl_2$ * $6H_2O$	0.32	g/L
Copper (II) sulfate heptahydrate	$CuSO_4$ * $5H_2O$	0.3	g/L
Sodium molybdate dihydrate	Na_2MoO_4 * $2H_2O$	0.4	g/L
Calcium chloride dihydrate	$CaCl_2$ * $2H_2O$	3	g/L
Iron (II) sulfate heptahydrate	$FeSO_4$ * $7H_2O$	3	g/L
Boric acid	H_3BO_3	1	g/L
Potassium iodide	KI	0.1	g/L

Table 4 - Vitamin Stock Solution

Component	Formula	Concentration	Units
d-Biotin	$C_{10}H_{16}N_2O_3S$	50	mg/L
Calcium pantothenate	$C_{18}H_{32}CaN_2O_{10}$	1000	mg/L
Nicotinic acid	$C_6H_5NO_2$	1000	mg/L
Thiamine hydrochloride	$C_{12}H_{17}ClN_4OS \cdot HCl$	1000	mg/L
Pyridoxine hydrochloride	$C_8H_{11}NO_3 \cdot HCl$	1000	mg/L
p-aminobenzoic acid	$C_7H_7NO_2$	200	mg/L
myo-inositol	$C_6H_{12}O_6$	25000	mg/L

Table 5 - Fermentation Feed Medium

Component	Formula	Concentration	Units
Glucose monohydrate	$C_6H_{12}O_6$* H_2O	660	g/L
Urea (in urea treatments only)	NH_2CONH_2	33	g/L
Antifoam		1.3	g/L
Potassium sulfate	K_2SO_4	4.2	g/L
Sodium sulfate	Na_2SO_4	0.336	g/L
Magnesium sulfate heptahydrate	$MgSO_4$ * $7H_2O$	6.12	g/L
Monobasic potassium phosphate	KH_2PO_4	10.8	g/L
Trace metal stock		14.4	mL/L
Vitamin stock		14.4	mL/L

Example 2

Enrichment or Release and Analysis of Steviol Glycosides

[0086] The fermentation broth generated from a recombinant yeast fermentation as described in Example 1 was used. The measurement of total steviol glycosides including intracellular and extracellular concentrations as well as the total nitrogen content (intracellular and extracellular) and total phosphorous content (intracellular and extracellular) was carried out by agitating the fermentation samples including the yeast cells to ensure that yeast cells were mixed and did not settle to the bottom of the vial. 100 μL of the mixed fermentation broth were pipetted into a 2 mL microcentrifuge tube. 900 μL of 61% methanol (extraction solvent) was added into the 2 ml microcentrifuge tube and agitated by placing on a sample rotator for 10 min to extract the steviol glycosides. The samples were then centrifuged at 10K rpm in a microcentrifuge for 3 min and the clarified supernatant was pipetted into an autosampler vial for analysis.

[0087] The fermentation broth (including cells) was heated at various temperatures and for various times. Heating was accomplished using a hot water bath and the sample was prepared as follows: 1 ml of the fermentation broth (including cells) was placed into a headspace vial for each temperature and time point studied. The vials were sealed and placed into the water bath and allowed to equilibrate for 5 minutes at each temperature studied prior to maintaining the samples at the desired temperature for the desired amount of time. At the end of the designated time, the samples were removed from the water bath and were cooled at 4°C. 4 ml of sterile water was added to each vial after which the sample was transferred to a 14 ml sterile tube. Samples were vortexed and 1 ml was removed for determining cell viability by the plating method. The remainder of the sample was centrifuged at 4700 rpm for 30 minutes at room temperature to obtain clear, cell-free supernatant. The cell-free supernatant was removed for further analysis, for example the extracellular content of steviol glycosides, nitrogen, and phosphorus in the fermentation medium (Phosphorous via ICP, Nitrogen via Antek and glycoside via UHPLC).

[0088] The amount of glycosides released into the fermentation broth after the heat treatment was determined. Reb A, Reb D and Reb M were measured by ultra-performance liquid chromatography (UPLC). The cell free supernatant was also used to evaluate nitrogen and phosphorous release into the fermentation broth. Nitrogen levels were analyzed using the combustion method and an ANTEK™ nitrogen analyzer (Antek 9000 nitrogen detector utilizing nitrogen combustion and reaction with ozone to produce metastable nitrogen dioxide, which is detected via chemiluminescence and phosphorous levels were analyzed using inductively coupled plasma spectrometry ICP, Perkin Elmer Optima 3200DV, UV detection at 213.617nm, samples dissolved in 0.5% HNO_3). Yeast viability was determined using the Potato Dextrose Antibiotic Easy gel plates (Microbiology Labs test kits), incubated at room temperature for 5 days. UPLC Method for glycoside separation

[0089] The steviol glycosides were separated using an Eclipse C18 Plus RRHD column (3mm x 150mm x 1.8um) with Eclipse Plus C18 guard (column 3mm x 5mm x 1.8um). The mobile phase used was channel A: 10mM phosphate buffer, pH 2.6 and channel B acetonitrile. The flow rate was 0.6 ml/min, the column temperature was 40° C and the detection was performed at ultraviolet absorption of 210 nm. The gradient elution profile in which the ratio of phosphate buffer to acetonitrile changed is shown below:

i. Initial: 80% A/20% B
ii. 7 minutes: 70% A/30% B
iii. 12 minutes: 70% A/30% B
iv. 15 minutes: 45% A/55% B
v. 18 minutes: 45% A/55% B

vi. 20 minutes: 20% A/80% B
vii. 25 minutes: 20% A/80% B
viii. 27 minutes: 15% A/85% B
ix. 30 minutes: 15% A/85% B
x. 30.5 minutes: 80% A/20% B
xi. 35 minutes: 80% A/20% B

[0090] The UPLC results, shown as the % of extracellular Reb A, Reb D & M released from the yeast cell after the heat treatment is shown in Table 6. The % of the extracellular nitrogen and phosphorus are also shown.

[0091] Total nitrogen in the fermentation broth including cells was determined using a Leco Nitrogen Analyzer (Leco TruMac N, St. Joseph, MI). 1 ml of homogeneously mixed fermentation broth including cells were applied to a nickel lined ceramic combustion container for nitrogen combustion analysis.

[0092] Total phosphorus in the fermentation broth including cells was determined by ashing 5 mL of homogenously mixed fermentation broth with a propane torch in a ceramic crucible. The resultant ash was held at 525°C overnight prior to dissolving the ash with 10 mls of 0.5% HNO_3. Phosphorus content in the nitric acid solution was determined using ICP.

Table 6.

Heat treatment condition		**% extracellular Reb D&M**	**% extracellular Reb A**	**% extracellular Reb B**	**Reb B (% extracellular)**	**% extracellular N**	**% extracellular P**
Temperature, °C	Time hr						
24	1	34%	48%	20%	28%	20%	45%
	2	34%	48%	21%	28%	21%	45%
	24	43%	52%	25%	30%	25%	51%
	48	47%	58%	30%	34%	30%	56%
	70	48%	59%	31%	34%	31%	55%

Temperature, °C	Time, hr	% extracellular D&M	% extracellular A	% extracellular) B	% extracellular N	% extracellular P
50	0.08	44%	54%	30%	23%	46%
	0.5	56%	67%	36%	28%	55%
	1	72%	88%	41%	37%	65%
	1.5	76%	85%	44%	40%	67%
	2	88%	91%	46%	50%	76%
	24	98%	99%	58%	88%	93%
	48	95%	97%	60%	91%	92%
	70	93%	96%	61%	91%	97%
	72	92%	96%	60%	93%	95%
	96	91%	96%		91%	95%
	120	87%	92%		92%	95%

Temperature, °C	Time, hr	% extracellular D&M	Reb B (% extracellular)	% extracellular N	% extracellular P
70	0.08	101%		36%	64%
	0.25	101%	45%	35%	62%
	0.5	101%		36%	64%
	0.75	99%	45%	35%	66%
	1	101%		36%	66%
	1.5	101%	46%	37%	65%
	2	99%		37%	64%

Temperature, °C	Time, hr	% extracellular D&M	% extracellular A	Reb B (% extracellular)	% extracellular N	% extracellular P
75	0.08	96%	92%	44%	35%	65%
	0.5	98%	94%	45%	36%	68%
	1	96%	94%	46%	35%	69%
	2	95%	93%	47%	36%	67%
	24	80%	82%	69%	38%	74%
	48	74%	78%	86%	40%	82%
	70	68%	72%	98%	44%	88%

Temperature, °C	Time, hr	% extracellular D&M	% extracellular A	Reb B (% extracellular)	% extracellular N	% extracellular P
95	0.08	98%	93%	47%	32%	65%
	0.25	100%	99%	49%	33%	65%
	0.5	101%	99%	53%	36%	66%
	1	96%	91%	55%	35%	67%

[0093] As can be seen from Table 6, a heat treatment at a higher temperature and for a shorter time results in an increase in the release of the Reb A, Reb D and Reb M from within the yeast cell while affecting a heat kill but with

minimal cell lysis as evaluated by the amount of nitrogen and phosphorus in the medium after the heat treatment. The table also shows that at higher temperatures (50°C, 75°C and 95°C and longer times), Reb DM and RebA can degrade to Reb B resulting in a lower Reb DM content in the broth and an increase in Reb B content.

[0094] Viable yeast counts after heat treatment are shown in Table 7. It takes between 2 and 24 hours at 50°C to kill the yeast while no more than 5 minutes at 75°C or 95°C to kill the yeast.

Table 7

Temperature	Time	YM (CFU/ml)
24°**C**	24 h	1.02E+09
50°**C**	1h	2.03E+04
	2h	600
	24h	< 10
	48h	< 10
	72h	< 10
75°**C**	0.08 h	< 10
	0.5h	< 10
	1h	< 10
	2h	< 10
	24h	< 10
	48h	< 10
	70h	< 10
95°**C**	0.08h	< 10
	1h	< 10

Example 3

[0095] Fermentation broth from a strain designated A in a two-phase process was heat treated at 75°C for one hour. The two phase rates of feeding are shown below:

Strain	**pH control**	**Phase I**	**Phase II**
B	5.0	0.12u	0.166 mL/min

[0096] Yeast cells were stained by the fluorescent dyes based on the Nexcelom Cellometer method. Live cells before heat treatment appeared green under microscope and dead cells after heat treatment appeared red. Bright field microscopy images showed a lack of debris after heat treatment (compare FIG. 5A with FIG. 5B) indicating that the yeast cells were intact even after heat treatment.

SEQUENCE LISTING

[0097]

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            20                  25                  30

Arg Leu Ile Ser Lys Gly Val Lys Thr Thr Leu Val Thr Thr Ile His
        35                  40                  45

Thr Leu Asn Ser Thr Leu Asn His Ser Asn Thr Thr Thr Thr Ser Ile
    50                  55                  60

Glu Ile Gln Ala Ile Ser Asp Gly Cys Asp Glu Gly Gly Phe Met Ser
65                  70                  75                  80

Ala Gly Glu Ser Tyr Leu Glu Thr Phe Lys Gln Val Gly Ser Lys Ser
                85                  90                  95

Leu Ala Asp Leu Ile Lys Lys Leu Gln Ser Glu Gly Thr Thr Ile Asp
            100                 105                 110

Ala Ile Ile Tyr Asp Ser Met Thr Glu Trp Val Leu Asp Val Ala Ile
        115                 120                 125

Glu Phe Gly Ile Asp Gly Gly Ser Phe Phe Thr Gln Ala Cys Val Val
    130                 135                 140

Asn Ser Leu Tyr Tyr His Val His Lys Gly Leu Ile Ser Leu Pro Leu
145                 150                 155                 160

Gly Glu Thr Val Ser Val Pro Gly Phe Pro Val Leu Gln Arg Trp Glu
                165                 170                 175
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```
Thr Pro Leu Ile Leu Gln Asn His Glu Gln Ile Gln Ser Pro Trp Ser
            180                 185                 190

Gln Met Leu Phe Gly Gln Phe Ala Asn Ile Asp Gln Ala Arg Trp Val
        195                 200                 205

Phe Thr Asn Ser Phe Tyr Lys Leu Glu Glu Glu Val Ile Glu Trp Thr
    210                 215                 220

Arg Lys Ile Trp Asn Leu Lys Val Ile Gly Pro Thr Leu Pro Ser Met
225                 230                 235                 240

Tyr Leu Asp Lys Arg Leu Asp Asp Asp Lys Asp Asn Gly Phe Asn Leu
                245                 250                 255

Tyr Lys Ala Asn His His Glu Cys Met Asn Trp Leu Asp Asp Lys Pro
            260                 265                 270

Lys Glu Ser Val Val Tyr Val Ala Phe Gly Ser Leu Val Lys His Gly
        275                 280                 285

Pro Glu Gln Val Glu Glu Ile Thr Arg Ala Leu Ile Asp Ser Asp Val
    290                 295                 300

Asn Phe Leu Trp Val Ile Lys His Lys Glu Glu Gly Lys Leu Pro Glu
305                 310                 315                 320

Asn Leu Ser Glu Val Ile Lys Thr Gly Lys Gly Leu Ile Val Ala Trp
                325                 330                 335

Cys Lys Gln Leu Asp Val Leu Ala His Glu Ser Val Gly Cys Phe Val
            340                 345                 350

Thr His Cys Gly Phe Asn Ser Thr Leu Glu Ala Ile Ser Leu Gly Val
        355                 360                 365

Pro Val Val Ala Met Pro Gln Phe Ser Asp Gln Thr Thr Asn Ala Lys
    370                 375                 380

Leu Leu Asp Glu Ile Leu Gly Val Gly Val Arg Val Lys Ala Asp Glu
385                 390                 395                 400

Asn Gly Ile Val Arg Arg Gly Asn Leu Ala Ser Cys Ile Lys Met Ile
                405                 410                 415

Met Glu Glu Glu Arg Gly Val Ile Ile Arg Lys Asn Ala Val Lys Trp
            420                 425                 430
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Lys Asp Leu Ala Lys Val Ala Val His Glu Gly Gly Ser Ser Asp Asn
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35 40 45

Ile His Asn Gln Phe Leu Glu Ser Ser Gly Pro His Cys Leu Asp Gly
50 55 60

Ala Pro Gly Phe Arg Phe Glu Thr Ile Pro Asp Gly Val Ser His Ser
65 70 75 80

Pro Glu Ala Ser Ile Pro Ile Arg Glu Ser Leu Leu Arg Ser Ile Glu
85 90 95

Thr Asn Phe Leu Asp Arg Phe Ile Asp Leu Val Thr Lys Leu Pro Asp
100 105 110

Pro Pro Thr Cys Ile Ile Ser Asp Gly Phe Leu Ser Val Phe Thr Ile
115 120 125

Asp Ala Ala Lys Lys Leu Gly Ile Pro Val Met Met Tyr Trp Thr Leu
130 135 140

Ala Ala Cys Gly Phe Met Gly Phe Tyr His Ile His Ser Leu Ile Glu
145 150 155 160

Lys Gly Phe Ala Pro Leu Lys Asp Ala Ser Tyr Leu Thr Asn Gly Tyr
165 170 175

Leu Asp Thr Val Ile Asp Trp Val Pro Gly Met Glu Gly Ile Arg Leu
180 185 190

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Lys Asp Phe Pro Leu Asp Trp Ser Thr Asp Leu Asn Asp Lys Val Leu
        195                 200                 205

Met Phe Thr Thr Glu Ala Pro Gln Arg Ser His Lys Val Ser His His
    210                 215                 220

Ile Phe His Thr Phe Asp Glu Leu Glu Pro Ser Ile Ile Lys Thr Leu
225                 230                 235                 240

Ser Leu Arg Tyr Asn His Ile Tyr Thr Ile Gly Pro Leu Gln Leu Leu
                245                 250                 255

Leu Asp Gln Ile Pro Glu Glu Lys Lys Gln Thr Gly Ile Thr Ser Leu
            260                 265                 270

His Gly Tyr Ser Leu Val Lys Glu Glu Pro Glu Cys Phe Gln Trp Leu
        275                 280                 285

Gln Ser Lys Glu Pro Asn Ser Val Val Tyr Val Asn Phe Gly Ser Thr
    290                 295                 300

Thr Val Met Ser Leu Glu Asp Met Thr Glu Phe Gly Trp Gly Leu Ala
305                 310                 315                 320

Asn Ser Asn His Tyr Phe Leu Trp Ile Ile Arg Ser Asn Leu Val Ile
                325                 330                 335

Gly Glu Asn Ala Val Leu Pro Pro Glu Leu Glu Glu His Ile Lys Lys
            340                 345                 350

Arg Gly Phe Ile Ala Ser Trp Cys Ser Gln Glu Lys Val Leu Lys His
        355                 360                 365

Pro Ser Val Gly Gly Phe Leu Thr His Cys Gly Trp Gly Ser Thr Ile
    370                 375                 380

Glu Ser Leu Ser Ala Gly Val Pro Met Ile Cys Trp Pro Tyr Ser Trp
385                 390                 395                 400

Asp Gln Leu Thr Asn Cys Arg Tyr Ile Cys Lys Glu Trp Glu Val Gly
                405                 410                 415

Leu Glu Met Gly Thr Lys Val Lys Arg Asp Glu Val Lys Arg Leu Val
            420                 425                 430

Gln Glu Leu Met Gly Glu Gly Gly His Lys Met Arg Asn Lys Ala Lys
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            20                  25                  30

Ala Asn Val Leu Tyr Ser Lys Gly Phe Ser Ile Thr Ile Phe His Thr
        35                  40                  45

Asn Phe Asn Lys Pro Lys Thr Ser Asn Tyr Pro His Phe Thr Phe Arg
    50                  55                  60

Phe Ile Leu Asp Asn Asp Pro Gln Asp Glu Arg Ile Ser Asn Leu Pro
65                  70                  75                  80

Thr His Gly Pro Leu Ala Gly Met Arg Ile Pro Ile Ile Asn Glu His
                85                  90                  95

Gly Ala Asp Glu Leu Arg Arg Glu Leu Glu Leu Leu Met Leu Ala Ser
            100                 105                 110

Glu Glu Asp Glu Glu Val Ser Cys Leu Ile Thr Asp Ala Leu Trp Tyr
        115                 120                 125

Phe Ala Gln Ser Val Ala Asp Ser Leu Asn Leu Arg Arg Leu Val Leu
    130                 135                 140

Met Thr Ser Ser Leu Phe Asn Phe His Ala His Val Ser Leu Pro Gln
145                 150                 155                 160

Phe Asp Glu Leu Gly Tyr Leu Asp Pro Asp Asp Lys Thr Arg Leu Glu
```

```
                    165                 170                 175

Glu Gln Ala Ser Gly Phe Pro Met Leu Lys Val Lys Asp Ile Lys Ser
            180                 185                 190

Ala Tyr Ser Asn Trp Gln Ile Leu Lys Glu Ile Leu Gly Lys Met Ile
        195                 200                 205

Lys Gln Thr Lys Ala Ser Ser Gly Val Ile Trp Asn Ser Phe Lys Glu
    210                 215                 220

Leu Glu Glu Ser Glu Leu Glu Thr Val Ile Arg Glu Ile Pro Ala Pro
225                 230                 235                 240

Ser Phe Leu Ile Pro Leu Pro Lys His Leu Thr Ala Ser Ser Ser Ser
                245                 250                 255

Leu Leu Asp His Asp Arg Thr Val Phe Gln Trp Leu Asp Gln Gln Pro
            260                 265                 270

Pro Ser Ser Val Leu Tyr Val Ser Phe Gly Ser Thr Ser Glu Val Asp
        275                 280                 285

Glu Lys Asp Phe Leu Glu Ile Ala Arg Gly Leu Val Asp Ser Lys Gln
    290                 295                 300

Ser Phe Leu Trp Val Val Arg Pro Gly Phe Val Lys Gly Ser Thr Trp
305                 310                 315                 320

Val Glu Pro Leu Pro Asp Gly Phe Leu Gly Glu Arg Gly Arg Ile Val
                325                 330                 335

Lys Trp Val Pro Gln Gln Glu Val Leu Ala His Gly Ala Ile Gly Ala
            340                 345                 350

Phe Trp Thr His Ser Gly Trp Asn Ser Thr Leu Glu Ser Val Cys Glu
        355                 360                 365

Gly Val Pro Met Ile Phe Ser Asp Phe Gly Leu Asp Gln Pro Leu Asn
    370                 375                 380

Ala Arg Tyr Met Ser Asp Val Leu Lys Val Gly Val Tyr Leu Glu Asn
385                 390                 395                 400

Gly Trp Glu Arg Gly Glu Ile Ala Asn Ala Ile Arg Arg Val Met Val
                405                 410                 415
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Asp Glu Glu Gly Glu Tyr Ile Arg Gln Asn Ala Arg Val Leu Lys Gln
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    450                 455
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Leu Ser Lys Leu Ile Ala Glu Lys Gly His Lys Val Ser Phe Leu Ser
        35                  40                  45

Thr Thr Arg Asn Ile Gln Arg Leu Ser Ser His Ile Ser Pro Leu Ile
    50                  55                  60

Asn Val Val Gln Leu Thr Leu Pro Arg Val Gln Glu Leu Pro Glu Asp
65                  70                  75                  80

Ala Glu Ala Thr Thr Asp Val His Pro Glu Asp Ile Pro Tyr Leu Lys
                85                  90                  95

Lys Ala Ser Asp Gly Leu Gln Pro Glu Val Thr Arg Phe Leu Glu Gln
            100                 105                 110

His Ser Pro Asp Trp Ile Ile Tyr Asp Tyr Thr His Tyr Trp Leu Pro
        115                 120                 125

Ser Ile Ala Ala Ser Leu Gly Ile Ser Arg Ala His Phe Ser Val Thr
    130                 135                 140

Thr Pro Trp Ala Ile Ala Tyr Met Gly Pro Ser Ala Asp Ala Met Ile
145                 150                 155                 160

Asn Gly Ser Asp Gly Arg Thr Thr Val Glu Asp Leu Thr Thr Pro Pro
                165                 170                 175
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Lys Trp Phe Pro Phe Pro Thr Lys Val Cys Trp Arg Lys His Asp Leu
            180                 185                 190

Ala Arg Leu Val Pro Tyr Lys Ala Pro Gly Ile Ser Asp Gly Tyr Arg
        195                 200                 205

Met Gly Met Val Leu Lys Gly Ser Asp Cys Leu Leu Ser Lys Cys Tyr
    210                 215                 220

His Glu Phe Gly Thr Gln Trp Leu Pro Leu Leu Glu Thr Leu His Gln
225                 230                 235                 240

Val Pro Val Val Pro Val Gly Leu Leu Pro Pro Glu Ile Pro Gly Asp
                245                 250                 255

Glu Lys Asp Glu Thr Trp Val Ser Ile Lys Lys Trp Leu Asp Gly Lys
            260                 265                 270

Gln Lys Gly Ser Val Val Tyr Val Ala Leu Gly Ser Glu Ala Leu Val
        275                 280                 285

Ser Gln Thr Glu Val Val Glu Leu Ala Leu Gly Leu Glu Leu Ser Gly
    290                 295                 300

Leu Pro Phe Val Trp Ala Tyr Arg Lys Pro Lys Gly Pro Ala Lys Ser
305                 310                 315                 320

Asp Ser Val Glu Leu Pro Asp Gly Phe Val Glu Arg Thr Arg Asp Arg
                325                 330                 335

Gly Leu Val Trp Thr Ser Trp Ala Pro Gln Leu Arg Ile Leu Ser His
            340                 345                 350

Glu Ser Val Cys Gly Phe Leu Thr His Cys Gly Ser Gly Ser Ile Val
        355                 360                 365

Glu Gly Leu Met Phe Gly His Pro Leu Ile Met Leu Pro Ile Phe Gly
    370                 375                 380

Asp Gln Pro Leu Asn Ala Arg Leu Leu Glu Asp Lys Gln Val Gly Ile
385                 390                 395                 400

Glu Ile Pro Arg Asn Glu Glu Asp Gly Cys Leu Thr Lys Glu Ser Val
                405                 410                 415

Ala Arg Ser Leu Arg Ser Val Val Val Glu Lys Glu Gly Glu Ile Tyr
            420                 425                 430
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Lys Ala Asn Ala Arg Glu Leu Ser Lys Ile Tyr Asn Asp Thr Lys Val
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            20                  25                  30

Glu Arg Ile Tyr Glu Ala Met Arg Tyr Ser Leu Leu Ala Gly Gly Lys
        35                  40                  45

Arg Leu Arg Pro Ile Leu Cys Leu Ala Ala Cys Glu Leu Ala Gly Gly
    50                  55                  60

Ser Val Glu Gln Ala Met Pro Thr Ala Cys Ala Leu Glu Met Ile His
65                  70                  75                  80

Thr Met Ser Leu Ile His Asp Asp Leu Pro Ala Met Asp Asn Asp Asp
                85                  90                  95

Phe Arg Arg Gly Lys Pro Thr Asn His Lys Val Phe Gly Glu Asp Ile
            100                 105                 110

Ala Ile Leu Ala Gly Asp Ala Leu Leu Ala Tyr Ala Phe Glu His Ile
        115                 120                 125

Ala Ser Gln Thr Arg Gly Val Pro Pro Gln Leu Val Leu Gln Val Ile
    130                 135                 140

Ala Arg Ile Gly His Ala Val Ala Ala Thr Gly Leu Val Gly Gly Gln
145                 150                 155                 160

Val Val Asp Leu Glu Ser Glu Gly Lys Ala Ile Ser Leu Glu Thr Leu
                165                 170                 175

Glu Tyr Ile His Ser His Lys Thr Gly Ala Leu Leu Glu Ala Ser Val
            180                 185                 190
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Val Ser Gly Gly Ile Leu Ala Gly Ala Asp Glu Glu Leu Leu Ala Arg
        195                 200                 205

Leu Ser His Tyr Ala Arg Asp Ile Gly Leu Ala Phe Gln Ile Val Asp
    210                 215                 220

Asp Ile Leu Asp Val Thr Ala Thr Ser Glu Gln Leu Gly Lys Thr Ala
225                 230                 235                 240

Gly Lys Asp Gln Ala Ala Ala Lys Ala Thr Tyr Pro Ser Leu Leu Gly
                245                 250                 255

Leu Glu Ala Ser Arg Gln Lys Ala Glu Glu Leu Ile Gln Ser Ala Lys
            260                 265                 270

Glu Ala Leu Arg Pro Tyr Gly Ser Gln Ala Glu Pro Leu Leu Ala Leu
        275                 280                 285

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    290                 295
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            20                  25                  30

Asp Thr Val Ala Val Pro Ala Ala Ala Gly Arg Trp Arg Arg Ala Leu
        35                  40                  45

Ala Arg Ala Gln His Thr Ser Glu Ser Ala Ala Val Ala Lys Gly Ser
    50                  55                  60

Ser Leu Thr Pro Ile Val Arg Thr Asp Ala Glu Ser Arg Arg Thr Arg
65                  70                  75                  80

Trp Pro Thr Asp Asp Asp Asp Ala Glu Pro Leu Val Asp Glu Ile Arg
                85                  90                  95

Ala Met Leu Thr Ser Met Ser Asp Gly Asp Ile Ser Val Ser Ala Tyr
            100                 105                 110
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Asp Thr Ala Trp Val Gly Leu Val Pro Arg Leu Asp Gly Gly Glu Gly
        115                 120                 125

Pro Gln Phe Pro Ala Ala Val Arg Trp Ile Arg Asn Asn Gln Leu Pro
    130                 135                 140

Asp Gly Ser Trp Gly Asp Ala Ala Leu Phe Ser Ala Tyr Asp Arg Leu
145                 150                 155                 160

Ile Asn Thr Leu Ala Cys Val Val Thr Leu Thr Arg Trp Ser Leu Glu
                165                 170                 175

Pro Glu Met Arg Gly Arg Gly Leu Ser Phe Leu Gly Arg Asn Met Trp
            180                 185                 190

Lys Leu Ala Thr Glu Asp Glu Glu Ser Met Pro Ile Gly Phe Glu Leu
        195                 200                 205

Ala Phe Pro Ser Leu Ile Glu Leu Ala Lys Ser Leu Gly Val His Asp
    210                 215                 220

Phe Pro Tyr Asp His Gln Ala Leu Gln Gly Ile Tyr Ser Ser Arg Glu
225                 230                 235                 240

Ile Lys Met Lys Arg Ile Pro Lys Glu Val Met His Thr Val Pro Thr
                245                 250                 255

Ser Ile Leu His Ser Leu Glu Gly Met Pro Gly Leu Asp Trp Ala Lys
            260                 265                 270

Leu Leu Lys Leu Gln Ser Ser Asp Gly Ser Phe Leu Phe Ser Pro Ala
        275                 280                 285

Ala Thr Ala Tyr Ala Leu Met Asn Thr Gly Asp Asp Arg Cys Phe Ser
    290                 295                 300

Tyr Ile Asp Arg Thr Val Lys Lys Phe Asn Gly Gly Val Pro Asn Val
305                 310                 315                 320

Tyr Pro Val Asp Leu Phe Glu His Ile Trp Ala Val Asp Arg Leu Glu
                325                 330                 335

Arg Leu Gly Ile Ser Arg Tyr Phe Gln Lys Glu Ile Glu Gln Cys Met
            340                 345                 350

Asp Tyr Val Asn Arg His Trp Thr Glu Asp Gly Ile Cys Trp Ala Arg
        355                 360                 365
```

```
Asn Ser Asp Val Lys Glu Val Asp Asp Thr Ala Met Ala Phe Arg Leu
    370                 375                 380

Leu Arg Leu His Gly Tyr Ser Val Ser Pro Asp Val Phe Lys Asn Phe
385                 390                 395                 400

Glu Lys Asp Gly Glu Phe Phe Ala Phe Val Gly Gln Ser Asn Gln Ala
                405                 410                 415

Val Thr Gly Met Tyr Asn Leu Asn Arg Ala Ser Gln Ile Ser Phe Pro
            420                 425                 430

Gly Glu Asp Val Leu His Arg Ala Gly Ala Phe Ser Tyr Glu Phe Leu
        435                 440                 445

Arg Arg Lys Glu Ala Glu Gly Ala Leu Arg Asp Lys Trp Ile Ile Ser
    450                 455                 460

Lys Asp Leu Pro Gly Glu Val Val Tyr Thr Leu Asp Phe Pro Trp Tyr
465                 470                 475                 480

Gly Asn Leu Pro Arg Val Glu Ala Arg Asp Tyr Leu Glu Gln Tyr Gly
                485                 490                 495

Gly Gly Asp Asp Val Trp Ile Gly Lys Thr Leu Tyr Arg Met Pro Leu
            500                 505                 510

Val Asn Asn Asp Val Tyr Leu Glu Leu Ala Arg Met Asp Phe Asn His
        515                 520                 525

Cys Gln Ala Leu His Gln Leu Glu Trp Gln Gly Leu Lys Arg Trp Tyr
    530                 535                 540

Thr Glu Asn Arg Leu Met Asp Phe Gly Val Ala Gln Glu Asp Ala Leu
545                 550                 555                 560

Arg Ala Tyr Phe Leu Ala Ala Ala Ser Val Tyr Glu Pro Cys Arg Ala
                565                 570                 575

Ala Glu Arg Leu Ala Trp Ala Arg Ala Ala Ile Leu Ala Asn Ala Val
            580                 585                 590

Ser Thr His Leu Arg Asn Ser Pro Ser Phe Arg Glu Arg Leu Glu His
        595                 600                 605

Ser Leu Arg Cys Arg Pro Ser Glu Glu Thr Asp Gly Ser Trp Phe Asn
    610                 615                 620
```

```
Ser Ser Ser Gly Ser Asp Ala Val Leu Val Lys Ala Val Leu Arg Leu
625                 630                 635                 640

Thr Asp Ser Leu Ala Arg Glu Ala Gln Pro Ile His Gly Gly Asp Pro
                645                 650                 655

Glu Asp Ile Ile His Lys Leu Leu Arg Ser Ala Trp Ala Glu Trp Val
            660                 665                 670

Arg Glu Lys Ala Asp Ala Ala Asp Ser Val Cys Asn Gly Ser Ser Ala
        675                 680                 685

Val Glu Gln Glu Gly Ser Arg Met Val His Asp Lys Gln Thr Cys Leu
    690                 695                 700

Leu Leu Ala Arg Met Ile Glu Ile Ser Ala Gly Arg Ala Ala Gly Glu
705                 710                 715                 720

Ala Ala Ser Glu Asp Gly Asp Arg Arg Ile Ile Gln Leu Thr Gly Ser
                725                 730                 735

Ile Cys Asp Ser Leu Lys Gln Lys Met Leu Val Ser Gln Asp Pro Glu
            740                 745                 750

Lys Asn Glu Glu Met Met Ser His Val Asp Asp Glu Leu Lys Leu Arg
        755                 760                 765

Ile Arg Glu Phe Val Gln Tyr Leu Leu Arg Leu Gly Glu Lys Lys Thr
    770                 775                 780

Gly Ser Ser Glu Thr Arg Gln Thr Phe Leu Ser Ile Val Lys Ser Cys
785                 790                 795                 800

Tyr Tyr Ala Ala His Cys Pro Pro His Val Val Asp Arg His Ile Ser
                805                 810                 815

Arg Val Ile Phe Glu Pro Val Ser Ala Ala Lys
            820                 825
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<213> Arabidopsis thaliana

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Met Ser Ile Asn Leu Arg Ser Ser Gly Cys Ser Ser Pro Ile Ser Ala
1               5                   10                  15
```

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Thr Leu Glu Arg Gly Leu Asp Ser Glu Val Gln Thr Arg Ala Asn Asn
            20                  25                  30

Val Ser Phe Glu Gln Thr Lys Glu Lys Ile Arg Lys Met Leu Glu Lys
        35                  40                  45

Val Glu Leu Ser Val Ser Ala Tyr Asp Thr Ser Trp Val Ala Met Val
    50                  55                  60

Pro Ser Pro Ser Ser Gln Asn Ala Pro Leu Phe Pro Gln Cys Val Lys
65                  70                  75                  80

Trp Leu Leu Asp Asn Gln His Glu Asp Gly Ser Trp Gly Leu Asp Asn
                85                  90                  95

His Asp His Gln Ser Leu Lys Lys Asp Val Leu Ser Ser Thr Leu Ala
            100                 105                 110

Ser Ile Leu Ala Leu Lys Lys Trp Gly Ile Gly Glu Arg Gln Ile Asn
        115                 120                 125

Lys Gly Leu Gln Phe Ile Glu Leu Asn Ser Ala Leu Val Thr Asp Glu
    130                 135                 140

Thr Ile Gln Lys Pro Thr Gly Phe Asp Ile Ile Phe Pro Gly Met Ile
145                 150                 155                 160

Lys Tyr Ala Arg Asp Leu Asn Leu Thr Ile Pro Leu Gly Ser Glu Val
                165                 170                 175

Val Asp Asp Met Ile Arg Lys Arg Asp Leu Asp Leu Lys Cys Asp Ser
            180                 185                 190

Glu Lys Phe Ser Lys Gly Arg Glu Ala Tyr Leu Ala Tyr Val Leu Glu
        195                 200                 205

Gly Thr Arg Asn Leu Lys Asp Trp Asp Leu Ile Val Lys Tyr Gln Arg
    210                 215                 220

Lys Asn Gly Ser Leu Phe Asp Ser Pro Ala Thr Thr Ala Ala Ala Phe
225                 230                 235                 240

Thr Gln Phe Gly Asn Asp Gly Cys Leu Arg Tyr Leu Cys Ser Leu Leu
                245                 250                 255

Gln Lys Phe Glu Ala Ala Val Pro Ser Val Tyr Pro Phe Asp Gln Tyr
```

```
                260                 265                 270

Ala Arg Leu Ser Ile Ile Val Thr Leu Glu Ser Leu Gly Ile Asp Arg
        275                 280                 285

Asp Phe Lys Thr Glu Ile Lys Ser Ile Leu Asp Glu Thr Tyr Arg Tyr
    290                 295                 300

Trp Leu Arg Gly Asp Glu Glu Ile Cys Leu Asp Leu Ala Thr Cys Ala
305                 310                 315                 320

Leu Ala Phe Arg Leu Leu Leu Ala His Gly Tyr Asp Val Ser Tyr Asp
                325                 330                 335

Pro Leu Lys Pro Phe Ala Glu Glu Ser Gly Phe Ser Asp Thr Leu Glu
            340                 345                 350

Gly Tyr Val Lys Asn Thr Phe Ser Val Leu Glu Leu Phe Lys Ala Ala
        355                 360                 365

Gln Ser Tyr Pro His Glu Ser Ala Leu Lys Lys Gln Cys Cys Trp Thr
    370                 375                 380

Lys Gln Tyr Leu Glu Met Glu Leu Ser Ser Trp Val Lys Thr Ser Val
385                 390                 395                 400

Arg Asp Lys Tyr Leu Lys Lys Glu Val Glu Asp Ala Leu Ala Phe Pro
                405                 410                 415

Ser Tyr Ala Ser Leu Glu Arg Ser Asp His Arg Arg Lys Ile Leu Asn
            420                 425                 430

Gly Ser Ala Val Glu Asn Thr Arg Val Thr Lys Thr Ser Tyr Arg Leu
        435                 440                 445

His Asn Ile Cys Thr Ser Asp Ile Leu Lys Leu Ala Val Asp Asp Phe
    450                 455                 460

Asn Phe Cys Gln Ser Ile His Arg Glu Glu Met Glu Arg Leu Asp Arg
465                 470                 475                 480

Trp Ile Val Glu Asn Arg Leu Gln Glu Leu Lys Phe Ala Arg Gln Lys
                485                 490                 495

Leu Ala Tyr Cys Tyr Phe Ser Gly Ala Ala Thr Leu Phe Ser Pro Glu
            500                 505                 510
```

```
Leu Ser Asp Ala Arg Ile Ser Trp Ala Lys Gly Gly Val Leu Thr Thr
        515                 520                 525

Val Val Asp Asp Phe Phe Asp Val Gly Gly Ser Lys Glu Glu Leu Glu
    530                 535                 540

Asn Leu Ile His Leu Val Glu Lys Trp Asp Leu Asn Gly Val Pro Glu
545                 550                 555                 560

Tyr Ser Ser Glu His Val Glu Ile Ile Phe Ser Val Leu Arg Asp Thr
                565                 570                 575

Ile Leu Glu Thr Gly Asp Lys Ala Phe Thr Tyr Gln Gly Arg Asn Val
            580                 585                 590

Thr His His Ile Val Lys Ile Trp Leu Asp Leu Leu Lys Ser Met Leu
        595                 600                 605

Arg Glu Ala Glu Trp Ser Ser Asp Lys Ser Thr Pro Ser Leu Glu Asp
    610                 615                 620

Tyr Met Glu Asn Ala Tyr Ile Ser Phe Ala Leu Gly Pro Ile Val Leu
625                 630                 635                 640

Pro Ala Thr Tyr Leu Ile Gly Pro Pro Leu Pro Glu Lys Thr Val Asp
                645                 650                 655

Ser His Gln Tyr Asn Gln Leu Tyr Lys Leu Val Ser Thr Met Gly Arg
            660                 665                 670

Leu Leu Asn Asp Ile Gln Gly Phe Lys Arg Glu Ser Ala Glu Gly Lys
        675                 680                 685

Leu Asn Ala Val Ser Leu His Met Lys His Glu Arg Asp Asn Arg Ser
    690                 695                 700

Lys Glu Val Ile Ile Glu Ser Met Lys Gly Leu Ala Glu Arg Lys Arg
705                 710                 715                 720

Glu Glu Leu His Lys Leu Val Leu Glu Glu Lys Gly Ser Val Val Pro
                725                 730                 735

Arg Glu Cys Lys Glu Ala Phe Leu Lys Met Ser Lys Val Leu Asn Leu
            740                 745                 750

Phe Tyr Arg Lys Asp Asp Gly Phe Thr Ser Asn Asp Leu Met Ser Leu
        755                 760                 765
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Val Lys Ser Val Ile Tyr Glu Pro Val Ser Leu Gln Lys Glu Ser Leu
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Thr
785

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gagatagcca aggaggcatt ggtgaccaga ttccaatcca tatctacaag gaacttatct 360

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tatcataaaa cagttaagag acacatactg accgccgtct tgggtcctaa tgcacagaaa 480

aagcatagaa ttcacagaga tatcatgatg gataacatat ctactcaact tcatgaattc 540

gtgaaaaaca acccagaaca ggaagaggta gaccttagaa aaatctttca atctgagtta 600

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ctgaaaatca ctatgaatag agacgaaatc tttcaagtcc ttgttgttga tccaatgatg 720

ggagcaatcg atgttgattg gagagacttc tttccatacc taaagtgggt cccaaacaaa 780

aagttcgaaa atactattca acaaatgtac atcagaagag aagctgttat gaaatcttta 840

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<212> PRT
<213> Stevia rebaudiana

<400> 10

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Ile Gly Gly Thr Ala Val Ala Leu Ala Val Ala Leu Ile Phe Trp Tyr
            20                  25                  30

Leu Lys Ser Tyr Thr Ser Ala Arg Arg Ser Gln Ser Asn His Leu Pro
        35                  40                  45

Arg Val Pro Glu Val Pro Gly Val Pro Leu Leu Gly Asn Leu Leu Gln
    50                  55                  60

Leu Lys Glu Lys Lys Pro Tyr Met Thr Phe Thr Arg Trp Ala Ala Thr
65                  70                  75                  80

Tyr Gly Pro Ile Tyr Ser Ile Lys Thr Gly Ala Thr Ser Met Val Val
                85                  90                  95

Val Ser Ser Asn Glu Ile Ala Lys Glu Ala Leu Val Thr Arg Phe Gln
            100                 105                 110

Ser Ile Ser Thr Arg Asn Leu Ser Lys Ala Leu Lys Val Leu Thr Ala
        115                 120                 125

Asp Lys Thr Met Val Ala Met Ser Asp Tyr Asp Asp Tyr His Lys Thr
    130                 135                 140

Val Lys Arg His Ile Leu Thr Ala Val Leu Gly Pro Asn Ala Gln Lys
145                 150                 155                 160

Lys His Arg Ile His Arg Asp Ile Met Met Asp Asn Ile Ser Thr Gln
                165                 170                 175

Leu His Glu Phe Val Lys Asn Asn Pro Glu Gln Glu Glu Val Asp Leu
            180                 185                 190
```

```
Arg Lys Ile Phe Gln Ser Glu Leu Phe Gly Leu Ala Met Arg Gln Ala
        195                 200                 205

Leu Gly Lys Asp Val Glu Ser Leu Tyr Val Glu Asp Leu Lys Ile Thr
    210                 215                 220

Met Asn Arg Asp Glu Ile Phe Gln Val Leu Val Val Asp Pro Met Met
225                 230                 235                 240

Gly Ala Ile Asp Val Asp Trp Arg Asp Phe Phe Pro Tyr Leu Lys Trp
                245                 250                 255

Val Pro Asn Lys Lys Phe Glu Asn Thr Ile Gln Gln Met Tyr Ile Arg
            260                 265                 270

Arg Glu Ala Val Met Lys Ser Leu Ile Lys Glu His Lys Lys Arg Ile
        275                 280                 285

Ala Ser Gly Glu Lys Leu Asn Ser Tyr Ile Asp Tyr Leu Leu Ser Glu
    290                 295                 300

Ala Gln Thr Leu Thr Asp Gln Gln Leu Leu Met Ser Leu Trp Glu Pro
305                 310                 315                 320

Ile Ile Glu Ser Ser Asp Thr Thr Met Val Thr Thr Glu Trp Ala Met
                325                 330                 335

Tyr Glu Leu Ala Lys Asn Pro Lys Leu Gln Asp Arg Leu Tyr Arg Asp
            340                 345                 350

Ile Lys Ser Val Cys Gly Ser Glu Lys Ile Thr Glu Glu His Leu Ser
        355                 360                 365

Gln Leu Pro Tyr Ile Thr Ala Ile Phe His Glu Thr Leu Arg Arg His
    370                 375                 380

Ser Pro Val Pro Ile Ile Pro Leu Arg His Val His Glu Asp Thr Val
385                 390                 395                 400

Leu Gly Gly Tyr His Val Pro Ala Gly Thr Glu Leu Ala Val Asn Ile
                405                 410                 415

Tyr Gly Cys Asn Met Asp Lys Asn Val Trp Glu Asn Pro Glu Glu Trp
            420                 425                 430

Asn Pro Glu Arg Phe Met Lys Glu Asn Glu Thr Ile Asp Phe Gln Lys
        435                 440                 445
```

Thr Met Ala Phe Gly Gly Gly Lys Arg Val Cys Ala Gly Ser Leu Gln
450 455 460

Ala Leu Leu Thr Ala Ser Ile Gly Ile Gly Arg Met Val Gln Glu Phe
465 470 475 480

Glu Trp Lys Leu Lys Asp Met Thr Gln Glu Glu Val Asn Thr Ile Gly
485 490 495

Leu Thr Thr Gln Met Leu Arg Pro Leu Arg Ala Ile Ile Lys Pro Arg
500 505 510

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aaagccttag gtgaagaagc taaggcaaga tacgaaaaga ctagattcaa gatagtcgat 420

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gatattttgg tcgaacaagg tgctcaaaga ttagtccaag taggtttggg tgacgatgac 720

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<212> PRT
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Ala Ile Ile Lys Gly Glu Pro Val Ile Val Ser Asp Pro Ala Asn Ala
            20                  25                  30

Ser Ala Tyr Glu Ser Val Ala Ala Glu Leu Ser Ser Met Leu Ile Glu
        35                  40                  45

Asn Arg Gln Phe Ala Met Ile Val Thr Thr Ser Ile Ala Val Leu Ile
    50                  55                  60

Gly Cys Ile Val Met Leu Val Trp Arg Arg Ser Gly Ser Gly Asn Ser
65                  70                  75                  80
```

```
Lys Arg Val Glu Pro Leu Lys Pro Leu Val Ile Lys Pro Arg Glu Glu
                85                  90                  95

Glu Ile Asp Asp Gly Arg Lys Lys Val Thr Ile Phe Phe Gly Thr Gln
            100                 105                 110

Thr Gly Thr Ala Glu Gly Phe Ala Lys Ala Leu Gly Glu Glu Ala Lys
        115                 120                 125

Ala Arg Tyr Glu Lys Thr Arg Phe Lys Ile Val Asp Leu Asp Asp Tyr
    130                 135                 140

Ala Ala Asp Asp Asp Glu Tyr Glu Glu Lys Leu Lys Lys Glu Asp Val
145                 150                 155                 160

Ala Phe Phe Phe Leu Ala Thr Tyr Gly Asp Gly Glu Pro Thr Asp Asn
                165                 170                 175

Ala Ala Arg Phe Tyr Lys Trp Phe Thr Glu Gly Asn Asp Arg Gly Glu
            180                 185                 190

Trp Leu Lys Asn Leu Lys Tyr Gly Val Phe Gly Leu Gly Asn Arg Gln
        195                 200                 205

Tyr Glu His Phe Asn Lys Val Ala Lys Val Val Asp Asp Ile Leu Val
    210                 215                 220

Glu Gln Gly Ala Gln Arg Leu Val Gln Val Gly Leu Gly Asp Asp Asp
225                 230                 235                 240

Gln Cys Ile Glu Asp Asp Phe Thr Ala Trp Arg Glu Ala Leu Trp Pro
                245                 250                 255

Glu Leu Asp Thr Ile Leu Arg Glu Glu Gly Asp Thr Ala Val Ala Thr
            260                 265                 270

Pro Tyr Thr Ala Ala Val Leu Glu Tyr Arg Val Ser Ile His Asp Ser
        275                 280                 285

Glu Asp Ala Lys Phe Asn Asp Ile Thr Leu Ala Asn Gly Asn Gly Tyr
    290                 295                 300

Thr Val Phe Asp Ala Gln His Pro Tyr Lys Ala Asn Val Ala Val Lys
305                 310                 315                 320

Arg Glu Leu His Thr Pro Glu Ser Asp Arg Ser Cys Ile His Leu Glu
                325                 330                 335
```

```
Phe Asp Ile Ala Gly Ser Gly Leu Thr Met Lys Leu Gly Asp His Val
            340                 345                 350

Gly Val Leu Cys Asp Asn Leu Ser Glu Thr Val Asp Glu Ala Leu Arg
        355                 360                 365

Leu Leu Asp Met Ser Pro Asp Thr Tyr Phe Ser Leu His Ala Glu Lys
    370                 375                 380

Glu Asp Gly Thr Pro Ile Ser Ser Ser Leu Pro Pro Pro Phe Pro Pro
385                 390                 395                 400

Cys Asn Leu Arg Thr Ala Leu Thr Arg Tyr Ala Cys Leu Leu Ser Ser
                405                 410                 415

Pro Lys Lys Ser Ala Leu Val Ala Leu Ala Ala His Ala Ser Asp Pro
            420                 425                 430

Thr Glu Ala Glu Arg Leu Lys His Leu Ala Ser Pro Ala Gly Lys Asp
        435                 440                 445

Glu Tyr Ser Lys Trp Val Val Glu Ser Gln Arg Ser Leu Leu Glu Val
    450                 455                 460

Met Ala Glu Phe Pro Ser Ala Lys Pro Pro Leu Gly Val Phe Phe Ala
465                 470                 475                 480

Gly Val Ala Pro Arg Leu Gln Pro Arg Phe Tyr Ser Ile Ser Ser Ser
                485                 490                 495

Pro Lys Ile Ala Glu Thr Arg Ile His Val Thr Cys Ala Leu Val Tyr
            500                 505                 510

Glu Lys Met Pro Thr Gly Arg Ile His Lys Gly Val Cys Ser Thr Trp
        515                 520                 525

Met Lys Asn Ala Val Pro Tyr Glu Lys Ser Glu Lys Leu Phe Leu Gly
    530                 535                 540

Arg Pro Ile Phe Val Arg Gln Ser Asn Phe Lys Leu Pro Ser Asp Ser
545                 550                 555                 560

Lys Val Pro Ile Ile Met Ile Gly Pro Gly Thr Gly Leu Ala Pro Phe
                565                 570                 575

Arg Gly Phe Leu Gln Glu Arg Leu Ala Leu Val Glu Ser Gly Val Glu
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```
                    580                 585                 590

Leu Gly Pro Ser Val Leu Phe Phe Gly Cys Arg Asn Arg Arg Met Asp
        595                 600                 605

Phe Ile Tyr Glu Glu Glu Leu Gln Arg Phe Val Glu Ser Gly Ala Leu
    610                 615                 620

Ala Glu Leu Ser Val Ala Phe Ser Arg Glu Gly Pro Thr Lys Glu Tyr
625                 630                 635                 640

Val Gln His Lys Met Met Asp Lys Ala Ser Asp Ile Trp Asn Met Ile
                645                 650                 655

Ser Gln Gly Ala Tyr Leu Tyr Val Cys Gly Asp Ala Lys Gly Met Ala
            660                 665                 670

Arg Asp Val His Arg Ser Leu His Thr Ile Ala Gln Glu Gln Gly Ser
        675                 680                 685

Met Asp Ser Thr Lys Ala Glu Gly Phe Val Lys Asn Leu Gln Thr Ser
    690                 695                 700

Gly Arg Tyr Leu Arg Asp Val Trp
705                 710
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<212> PRT
<213> Oryza sativa

<400> 13

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Val Val Ile Cys Pro Trp Leu Ala Phe Gly His Leu Leu Pro Cys Leu
            20                  25                  30

Asp Leu Ala Gln Arg Leu Ala Ser Arg Gly His Arg Val Ser Phe Val
        35                  40                  45

Ser Thr Pro Arg Asn Ile Ser Arg Leu Pro Pro Val Arg Pro Ala Leu
    50                  55                  60

Ala Pro Leu Val Ala Phe Val Ala Leu Pro Leu Pro Arg Val Glu Gly
65                  70                  75                  80

Leu Pro Asp Gly Ala Glu Ser Thr Asn Asp Val Pro His Asp Arg Pro
```

```
                    85                  90                  95

Asp Met Val Glu Leu His Arg Arg Ala Phe Asp Gly Leu Ala Ala Pro
             100                 105                 110

Phe Ser Glu Phe Leu Gly Thr Ala Cys Ala Asp Trp Val Ile Val Asp
         115                 120                 125

Val Phe His His Trp Ala Ala Ala Ala Ala Leu Glu His Lys Val Pro
     130                 135                 140

Cys Ala Met Met Leu Leu Gly Ser Ala His Met Ile Ala Ser Ile Ala
145                 150                 155                 160

Asp Arg Arg Leu Glu Arg Ala Glu Thr Glu Ser Pro Ala Ala Ala Gly
                165                 170                 175

Gln Gly Arg Pro Ala Ala Ala Pro Thr Phe Glu Val Ala Arg Met Lys
             180                 185                 190

Leu Ile Arg Thr Lys Gly Ser Ser Gly Met Ser Leu Ala Glu Arg Phe
         195                 200                 205

Ser Leu Thr Leu Ser Arg Ser Ser Leu Val Val Gly Arg Ser Cys Val
     210                 215                 220

Glu Phe Glu Pro Glu Thr Val Pro Leu Leu Ser Thr Leu Arg Gly Lys
225                 230                 235                 240

Pro Ile Thr Phe Leu Gly Leu Met Pro Pro Leu His Glu Gly Arg Arg
                245                 250                 255

Glu Asp Gly Glu Asp Ala Thr Val Arg Trp Leu Asp Ala Gln Pro Ala
             260                 265                 270

Lys Ser Val Val Tyr Val Ala Leu Gly Ser Glu Val Pro Leu Gly Val
         275                 280                 285

Glu Lys Val His Glu Leu Ala Leu Gly Leu Glu Leu Ala Gly Thr Arg
     290                 295                 300

Phe Leu Trp Ala Leu Arg Lys Pro Thr Gly Val Ser Asp Ala Asp Leu
305                 310                 315                 320

Leu Pro Ala Gly Phe Glu Glu Arg Thr Arg Gly Arg Gly Val Val Ala
                325                 330                 335
```

```
Thr Arg Trp Val Pro Gln Met Ser Ile Leu Ala His Ala Ala Val Gly
            340                 345                 350

Ala Phe Leu Thr His Cys Gly Trp Asn Ser Thr Ile Glu Gly Leu Met
        355                 360                 365

Phe Gly His Pro Leu Ile Met Leu Pro Ile Phe Gly Asp Gln Gly Pro
    370                 375                 380

Asn Ala Arg Leu Ile Glu Ala Lys Asn Ala Gly Leu Gln Val Ala Arg
385                 390                 395                 400

Asn Asp Gly Asp Gly Ser Phe Asp Arg Glu Gly Val Ala Ala Ala Ile
                405                 410                 415

Arg Ala Val Ala Val Glu Glu Glu Ser Ser Lys Val Phe Gln Ala Lys
            420                 425                 430

Ala Lys Lys Leu Gln Glu Ile Val Ala Asp Met Ala Cys His Glu Arg
        435                 440                 445

Tyr Ile Asp Gly Phe Ile Gln Gln Leu Arg Ser Tyr Lys Asp
    450                 455                 460
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<210> 14
<211> 1503
<212> DNA
<213> Artificial Sequence

<220>
<223> Codon-optimized stevia rebaudiana

<400> 14

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actcaactta gaaggaagag cgctaatcta ccaccaaccg tgtttccatc aataccaatc 120

attggacact tatacttact caaaaagcct ctttatagaa ctttagcaaa aattgccgct 180

aagtacggac caatactgca attacaactc ggctacagac gtgttctggt gatttcctca 240

ccatcagcag cagaagagtg ctttaccaat aacgatgtaa tcttcgcaaa tagacctaag 300

acattgtttg gcaaaatagt gggtggaaca tcccttggca gtttatccta cggcgatcaa 360

tggcgtaatc taaggagagt agcttctatc gaaatcctat cagttcatag gttgaacgaa 420

tttcatgata tcagagtgga tgagaacaga ttgttaatta gaaaacttag aagttcatct 480

tctcctgtta ctcttataac agtcttttat gctctaacat tgaacgtcat tatgagaatg 540

atctctggca aaagatattt cgacagtggg gatagagaat tggaggagga aggtaagaga 600

tttcgagaaa tcttagacga aacgttgctt ctagccggtg cttctaatgt tggcgactac 660

ttaccaatat tgaactggtt gggagttaag tctcttgaaa agaaattgat cgctttgcag 720

aaaaagagag atgacttttt ccagggtttg attgaacagg ttagaaaatc tcgtggtgct 780

aaagtaggca aaggtagaaa aacgatgatc gaactcttat tatctttgca agagtcagaa 840

cctgagtact atacagatgc tatgataaga tcttttgtcc taggtctgct ggctgcaggt 900

agtgatactt cagcgggcac tatggaatgg gccatgagct tactggtcaa tcacccacat 960

gtattgaaga aagctcaagc tgaaatcgat agagttatcg gtaataacag attgattgac 1020

gagtcagaca ttggaaatat cccttacatc gggtgtatta tcaatgaaac tctaagactc 1080

tatccagcag ggccattgtt gttcccacat gaaagttctg ccgactgcgt tatttccggt 1140

tacaatatac ctagaggtac aatgttaatc gtaaaccaat gggcgattca tcacgatcct 1200

aaagtctggg atgatcctga aacctttaaa cctgaaagat ttcaaggatt agaaggaact 1260

agagatggtt tcaaacttat gccattcggt tctgggagaa gaggatgtcc aggtgaaggt 1320

ttggcaataa ggctgttagg gatgacacta ggctcagtga tccaatgttt tgattgggag 1380

agagtaggag atgagatggt tgacatgaca gaaggtttgg gtgtcacact tcctaaggcc 1440

gttccattag ttgccaaatg taagccacgt tccgaaatga ctaatctcct atccgaactt 1500

taa 1503

<210> 15
<211> 500
<212> PRT
<213> Stevia rebaudiana

<400> 15

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Met Glu Ala Ser Tyr Leu Tyr Ile Ser Ile Leu Leu Leu Leu Ala Ser
1               5                  10                  15

Tyr Leu Phe Thr Thr Gln Leu Arg Arg Lys Ser Ala Asn Leu Pro Pro
            20                  25                  30

Thr Val Phe Pro Ser Ile Pro Ile Ile Gly His Leu Tyr Leu Leu Lys
        35                  40                  45

Lys Pro Leu Tyr Arg Thr Leu Ala Lys Ile Ala Ala Lys Tyr Gly Pro
    50                  55                  60

Ile Leu Gln Leu Gln Leu Gly Tyr Arg Arg Val Leu Val Ile Ser Ser
65                  70                  75                  80

Pro Ser Ala Ala Glu Glu Cys Phe Thr Asn Asn Asp Val Ile Phe Ala
                85                  90                  95

Asn Arg Pro Lys Thr Leu Phe Gly Lys Ile Val Gly Gly Thr Ser Leu
```

```
                100                 105                 110

Gly Ser Leu Ser Tyr Gly Asp Gln Trp Arg Asn Leu Arg Arg Val Ala
        115                 120                 125

Ser Ile Glu Ile Leu Ser Val His Arg Leu Asn Glu Phe His Asp Ile
    130                 135                 140

Arg Val Asp Glu Asn Arg Leu Leu Ile Arg Lys Leu Arg Ser Ser Ser
145                 150                 155                 160

Ser Pro Val Thr Leu Ile Thr Val Phe Tyr Ala Leu Thr Leu Asn Val
                165                 170                 175

Ile Met Arg Met Ile Ser Gly Lys Arg Tyr Phe Asp Ser Gly Asp Arg
            180                 185                 190

Glu Leu Glu Glu Glu Gly Lys Arg Phe Arg Glu Ile Leu Asp Glu Thr
        195                 200                 205

Leu Leu Leu Ala Gly Ala Ser Asn Val Gly Asp Tyr Leu Pro Ile Leu
    210                 215                 220

Asn Trp Leu Gly Val Lys Ser Leu Glu Lys Lys Leu Ile Ala Leu Gln
225                 230                 235                 240

Lys Lys Arg Asp Asp Phe Phe Gln Gly Leu Ile Glu Gln Val Arg Lys
                245                 250                 255

Ser Arg Gly Ala Lys Val Gly Lys Gly Arg Lys Thr Met Ile Glu Leu
            260                 265                 270

Leu Leu Ser Leu Gln Glu Ser Glu Pro Glu Tyr Tyr Thr Asp Ala Met
        275                 280                 285

Ile Arg Ser Phe Val Leu Gly Leu Leu Ala Ala Gly Ser Asp Thr Ser
    290                 295                 300

Ala Gly Thr Met Glu Trp Ala Met Ser Leu Leu Val Asn His Pro His
305                 310                 315                 320

Val Leu Lys Lys Ala Gln Ala Glu Ile Asp Arg Val Ile Gly Asn Asn
                325                 330                 335

Arg Leu Ile Asp Glu Ser Asp Ile Gly Asn Ile Pro Tyr Ile Gly Cys
            340                 345                 350
```

```
Ile Ile Asn Glu Thr Leu Arg Leu Tyr Pro Ala Gly Pro Leu Leu Phe
        355                 360                 365

Pro His Glu Ser Ser Ala Asp Cys Val Ile Ser Gly Tyr Asn Ile Pro
    370                 375                 380

Arg Gly Thr Met Leu Ile Val Asn Gln Trp Ala Ile His His Asp Pro
385                 390                 395                 400

Lys Val Trp Asp Asp Pro Glu Thr Phe Lys Pro Glu Arg Phe Gln Gly
                405                 410                 415

Leu Glu Gly Thr Arg Asp Gly Phe Lys Leu Met Pro Phe Gly Ser Gly
            420                 425                 430

Arg Arg Gly Cys Pro Gly Glu Gly Leu Ala Ile Arg Leu Leu Gly Met
        435                 440                 445

Thr Leu Gly Ser Val Ile Gln Cys Phe Asp Trp Glu Arg Val Gly Asp
    450                 455                 460

Glu Met Val Asp Met Thr Glu Gly Leu Gly Val Thr Leu Pro Lys Ala
465                 470                 475                 480

Val Pro Leu Val Ala Lys Cys Lys Pro Arg Ser Glu Met Thr Asn Leu
                485                 490                 495

Leu Ser Glu Leu
            500
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<210> 16
<211> 2124
<212> DNA
<213> Stevia rebaudiana

<400> 16

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aaggttttgg acacatcgaa cgcatcggaa tcgggagaat ctgctatgct gccgactata 120

gcgatgatta tggagaatcg tgagctgttg atgatactca caacgtcggt tgctgtattg 180

atcggatgcg ttgtcgtttt ggtgtggcgg agatcgtcta cgaagaagtc ggcgttggag 240

ccaccggtga ttgtggttcc gaagagagtg caagaggagg aagttgatga tggtaagaag 300

aaagttacgg ttttcttcgg cacccaaact ggaacagctg aaggcttcgc taaggcactt 360

gttgaggaag ctaaagctcg atatgaaaag gctgtcttta aagtaattga tttggatgat 420

tatgctgctg atgacgatga gtatgaggag aaactaaaga aagaatcttt ggccttttc 480

tttttggcta cgtatggaga tggtgagcca acagataatg ctgccagatt ttataaatgg 540

tttactgagg gagatgcgaa aggagaatgg cttaataagc ttcaatatgg agtatttggt 600

ttgggtaaca gacaatatga acattttaac aagatcgcaa aagtggttga tgatggtctt 660

gtagaacagg gtgcaaagcg tcttgttcct gttggacttg gagatgatga tcaatgtatt 720

gaagatgact tcaccgcatg gaaagagtta gtatggccgg agttggatca attacttcgt 780

gatgaggatg acacaactgt tgctactcca tacacagctg ctgttgcaga atatcgcgtt 840

gtttttcatg aaaaaccaga cgcgctttct gaagattata gttatacaaa tggccatgct 900

gttcatgatg ctcaacatcc atgcagatcc aacgtggctg tcaaaaagga acttcatagt 960

cctgaatctg accggtcttg cactcatctt gaatttgaca tctcgaacac cggactatca 1020

tatgaaactg gggaccatgt tggagtttac tgtgaaaact tgagtgaagt tgtgaatgat 1080

gctgaaagat tagtaggatt accaccagac acttactcct ccatccacac tgatagtgaa 1140

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aaagcattga cgtgttatgc tgatgttttg agttctccca agaagtcggc tttgcttgca 1260

ctagctgctc atgccaccga tcccagtgaa gctgatagat tgaaatttct tgcatccccc 1320

gccggaaagg atgaatattc tcaatggata gttgcaagcc aaagaagtct ccttgaagtc 1380

atggaagcat tcccgtcagc taagccttca cttggtgttt tctttgcatc tgttgccccg 1440

cgcttacaac caagatacta ctctatttct tcctcaccca agatggcacc ggataggatt 1500

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ccaatatacg tccgaacatc caatttcaga ctaccatctg accctaaggt cccggttatc 1680

atgattggac ctggcactgg tttggctcct tttagaggtt tccttcaaga gcggttagct 1740

ttaaaggaag ccggaactga cctcggttta tccattttat tcttcggatg taggaatcgc 1800

aaagtggatt tcatatatga aaacgagctt aacaactttg tggagactgg tgctctttct 1860

gagcttattg ttgctttctc ccgtgaaggc ccgactaagg aatatgtgca acacaagatg 1920

agtgagaagg cttcggatat ctggaacttg ctttctgaag gagcatattt atacgtatgt 1980

ggtgatgcca aaggcatggc caaagatgta catcgaaccc tccacacaat tgtgcaagaa 2040

cagggatctc ttgactcgtc aaaggcagaa ctctacgtga agaatctaca aatgtcagga 2100

agatacctcc gtgacgtttg gtaa 2124

<210> 17
<211> 707
<212> PRT
<213> Stevia rebaudiana

<400> 17

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Met Gln Ser Asn Ser Val Lys Ile Ser Pro Leu Asp Leu Val Thr Ala
1               5                   10                  15

Leu Phe Ser Gly Lys Val Leu Asp Thr Ser Asn Ala Ser Glu Ser Gly
            20                  25                  30

Glu Ser Ala Met Leu Pro Thr Ile Ala Met Ile Met Glu Asn Arg Glu
        35                  40                  45

Leu Leu Met Ile Leu Thr Thr Ser Val Ala Val Leu Ile Gly Cys Val
    50                  55                  60

Val Val Leu Val Trp Arg Arg Ser Ser Thr Lys Lys Ser Ala Leu Glu
65                  70                  75                  80

Pro Pro Val Ile Val Val Pro Lys Arg Val Gln Glu Glu Glu Val Asp
                85                  90                  95

Asp Gly Lys Lys Lys Val Thr Val Phe Phe Gly Thr Gln Thr Gly Thr
            100                 105                 110

Ala Glu Gly Phe Ala Lys Ala Leu Val Glu Glu Ala Lys Ala Arg Tyr
        115                 120                 125

Glu Lys Ala Val Phe Lys Val Ile Asp Leu Asp Asp Tyr Ala Ala Asp
    130                 135                 140

Asp Asp Glu Tyr Glu Glu Lys Leu Lys Lys Glu Ser Leu Ala Phe Phe
145                 150                 155                 160

Phe Leu Ala Thr Tyr Gly Asp Gly Glu Pro Thr Asp Asn Ala Ala Arg
                165                 170                 175

Phe Tyr Lys Trp Phe Thr Glu Gly Asp Ala Lys Gly Glu Trp Leu Asn
            180                 185                 190

Lys Leu Gln Tyr Gly Val Phe Gly Leu Gly Asn Arg Gln Tyr Glu His
        195                 200                 205

Phe Asn Lys Ile Ala Lys Val Val Asp Asp Gly Leu Val Glu Gln Gly
    210                 215                 220

Ala Lys Arg Leu Val Pro Val Gly Leu Gly Asp Asp Asp Gln Cys Ile
225                 230                 235                 240

Glu Asp Asp Phe Thr Ala Trp Lys Glu Leu Val Trp Pro Glu Leu Asp
                245                 250                 255
```

```
Gln Leu Leu Arg Asp Glu Asp Asp Thr Thr Val Ala Thr Pro Tyr Thr
            260                 265                 270

Ala Ala Val Ala Glu Tyr Arg Val Val Phe His Glu Lys Pro Asp Ala
        275                 280                 285

Leu Ser Glu Asp Tyr Ser Tyr Thr Asn Gly His Ala Val His Asp Ala
    290                 295                 300

Gln His Pro Cys Arg Ser Asn Val Ala Val Lys Lys Glu Leu His Ser
305                 310                 315                 320

Pro Glu Ser Asp Arg Ser Cys Thr His Leu Glu Phe Asp Ile Ser Asn
                325                 330                 335

Thr Gly Leu Ser Tyr Glu Thr Gly Asp His Val Gly Val Tyr Cys Glu
            340                 345                 350

Asn Leu Ser Glu Val Val Asn Asp Ala Glu Arg Leu Val Gly Leu Pro
        355                 360                 365

Pro Asp Thr Tyr Ser Ser Ile His Thr Asp Ser Glu Asp Gly Ser Pro
    370                 375                 380

Leu Gly Gly Ala Ser Leu Pro Pro Pro Phe Pro Pro Cys Thr Leu Arg
385                 390                 395                 400

Lys Ala Leu Thr Cys Tyr Ala Asp Val Leu Ser Ser Pro Lys Lys Ser
                405                 410                 415

Ala Leu Leu Ala Leu Ala Ala His Ala Thr Asp Pro Ser Glu Ala Asp
            420                 425                 430

Arg Leu Lys Phe Leu Ala Ser Pro Ala Gly Lys Asp Glu Tyr Ser Gln
        435                 440                 445

Trp Ile Val Ala Ser Gln Arg Ser Leu Leu Glu Val Met Glu Ala Phe
    450                 455                 460

Pro Ser Ala Lys Pro Ser Leu Gly Val Phe Phe Ala Ser Val Ala Pro
465                 470                 475                 480

Arg Leu Gln Pro Arg Tyr Tyr Ser Ile Ser Ser Ser Pro Lys Met Ala
                485                 490                 495

Pro Asp Arg Ile His Val Thr Cys Ala Leu Val Tyr Glu Lys Thr Pro
            500                 505                 510
```

```
Ala Gly Arg Ile His Lys Gly Val Cys Ser Thr Trp Met Lys Asn Ala
        515                 520                 525

Val Pro Met Thr Glu Ser Gln Asp Cys Ser Trp Ala Pro Ile Tyr Val
    530                 535                 540

Arg Thr Ser Asn Phe Arg Leu Pro Ser Asp Pro Lys Val Pro Val Ile
545                 550                 555                 560

Met Ile Gly Pro Gly Thr Gly Leu Ala Pro Phe Arg Gly Phe Leu Gln
                565                 570                 575

Glu Arg Leu Ala Leu Lys Glu Ala Gly Thr Asp Leu Gly Leu Ser Ile
            580                 585                 590

Leu Phe Phe Gly Cys Arg Asn Arg Lys Val Asp Phe Ile Tyr Glu Asn
        595                 600                 605

Glu Leu Asn Asn Phe Val Glu Thr Gly Ala Leu Ser Glu Leu Ile Val
    610                 615                 620

Ala Phe Ser Arg Glu Gly Pro Thr Lys Glu Tyr Val Gln His Lys Met
625                 630                 635                 640

Ser Glu Lys Ala Ser Asp Ile Trp Asn Leu Leu Ser Glu Gly Ala Tyr
                645                 650                 655

Leu Tyr Val Cys Gly Asp Ala Lys Gly Met Ala Lys Asp Val His Arg
            660                 665                 670

Thr Leu His Thr Ile Val Gln Glu Gln Gly Ser Leu Asp Ser Ser Lys
        675                 680                 685

Ala Glu Leu Tyr Val Lys Asn Leu Gln Met Ser Gly Arg Tyr Leu Arg
    690                 695                 700

Asp Val Trp
705
```

<210> 18
<211> 2133
<212> DNA
<213> Artificial Sequence

<220>
<223> Codon optimized

<400> 18

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aaggcaatgg aaaagttgaa cgctagtgaa tctgaagatc caacaacatt gcctgcacta 120

aagatgctag ttgaaaatag agaattgttg acactgttca caacttcctt cgcagttctt 180

attgggtgtc ttgtatttct aatgtggaga cgttcatcct ctaaaaagct ggtacaagat 240

ccagttccac aagttatcgt tgtaaagaag aaagagaagg agtcagaggt tgatgacggg 300

aaaaagaaag tttctatttt ctacggcaca caaacaggaa ctgccgaagg ttttgctaaa 360

gcattagtcg aggaagcaaa agtgagatat gaaaagacct ctttcaaggt tatcgatcta 420

gatgactacg ctgcagatga tgatgaatat gaggaaaaac tgaaaaagga atccttagcc 480

ttcttcttct tggccacata cggtgatggt gaacctactg ataatgctgc taacttctac 540

aagtggttca cagaaggcga cgataaaggt gaatggctga aaaagttaca atacggagta 600

tttggtttag gtaacagaca atatgaacat ttcaacaaga tcgctattgt agttgatgat 660

aaacttactg aaatgggagc caaaagatta gtaccagtag gattagggga tgatgatcag 720

tgtatagaag atgacttcac cgcctggaag gaattggtat ggccagaatt ggatcaactt 780

ttaagggacg aagatgatac ttctgtgact accccataca ctgcagccgt attggagtac 840

agagtggttt accatgataa accagcagac tcatatgctg aagatcaaac ccatacaaac 900

ggtcatgttg ttcatgatgc acagcatcct tcaagatcta atgtggcttt caaaaaggaa 960

ctacacacct ctcaatcaga taggtcttgt actcacttag aattcgatat ttctcacaca 1020

ggactgtctt acgaaactgg cgatcacgtt ggcgtttatt ccgagaactt gtccgaagtt 1080

gtcgatgaag cactaaaact gttagggtta tcaccagaca catacttctc agtccatgct 1140

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gcttcaccag ccggaaaaga tgaatatgca caatggatcg tcgccaacca acgttctttg 1380

ctagaagtga tgcaaagttt tccatctgcc aagcctccat taggtgtgtt cttcgcagca 1440

gtagctccac gtttacaacc aagatactac tctatcagtt catctcctaa gatgtctcct 1500

aacagaatac atgttacatg tgctttggtg tacgagacta ctccagcagg cagaattcac 1560

agaggattgt gttcaacctg gatgaaaaat gctgtccctt taacagagtc acctgattgc 1620

tctcaagcat ccattttcgt tagaacatca aatttcagac ttccagtgga tccaaaagtt 1680

ccagtcatta tgataggacc aggcactggt cttgccccat tcaggggctt tcttcaagag 1740

agattggcct tgaaggaatc tggtacagaa ttgggttctt ctatcttttt ctttggttgc 1800

cgtaatagaa aagttgactt tatctacgag gacgagctta acaattttgt tgagacagga 1860

gcattgtcag aattgatcgt cgcattttca agagaaggga ctgccaaaga gtacgttcag 1920

cacaagatga gtcaaaaagc ctccgatata tggaaacttc taagtgaagg tgcctatctt 1980

tatgtctgtg gcgatgcaaa gggcatggcc aaggatgtcc atagaactct gcatacaatt 2040

gttcaggaac aagggagtct ggattcttcc aaggctgaat tgtacgtcaa aaacttacag 2100

atgtctggaa gatacttaag agatgtttgg taa 2133

<210> 19
<211> 710
<212> PRT
<213> Stevia rebaudiana

<400> 19

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Met Gln Ser Asp Ser Val Lys Val Ser Pro Phe Asp Leu Val Ser Ala
1               5                  10                  15

Ala Met Asn Gly Lys Ala Met Glu Lys Leu Asn Ala Ser Glu Ser Glu
            20                  25                  30

Asp Pro Thr Thr Leu Pro Ala Leu Lys Met Leu Val Glu Asn Arg Glu
        35                  40                  45

Leu Leu Thr Leu Phe Thr Thr Ser Phe Ala Val Leu Ile Gly Cys Leu
    50                  55                  60

Val Phe Leu Met Trp Arg Arg Ser Ser Ser Lys Lys Leu Val Gln Asp
65                  70                  75                  80

Pro Val Pro Gln Val Ile Val Val Lys Lys Lys Glu Lys Glu Ser Glu
                85                  90                  95

Val Asp Asp Gly Lys Lys Lys Val Ser Ile Phe Tyr Gly Thr Gln Thr
            100                 105                 110

Gly Thr Ala Glu Gly Phe Ala Lys Ala Leu Val Glu Glu Ala Lys Val
        115                 120                 125

Arg Tyr Glu Lys Thr Ser Phe Lys Val Ile Asp Leu Asp Asp Tyr Ala
    130                 135                 140

Ala Asp Asp Asp Glu Tyr Glu Glu Lys Leu Lys Lys Glu Ser Leu Ala
145                 150                 155                 160

Phe Phe Phe Leu Ala Thr Tyr Gly Asp Gly Glu Pro Thr Asp Asn Ala
                165                 170                 175

Ala Asn Phe Tyr Lys Trp Phe Thr Glu Gly Asp Asp Lys Gly Glu Trp
            180                 185                 190
```

```
Leu Lys Lys Leu Gln Tyr Gly Val Phe Gly Leu Gly Asn Arg Gln Tyr
        195                 200                 205

Glu His Phe Asn Lys Ile Ala Ile Val Val Asp Asp Lys Leu Thr Glu
    210                 215                 220

Met Gly Ala Lys Arg Leu Val Pro Val Gly Leu Gly Asp Asp Asp Gln
225                 230                 235                 240

Cys Ile Glu Asp Asp Phe Thr Ala Trp Lys Glu Leu Val Trp Pro Glu
                245                 250                 255

Leu Asp Gln Leu Leu Arg Asp Glu Asp Asp Thr Ser Val Thr Thr Pro
            260                 265                 270

Tyr Thr Ala Ala Val Leu Glu Tyr Arg Val Val Tyr His Asp Lys Pro
        275                 280                 285

Ala Asp Ser Tyr Ala Glu Asp Gln Thr His Thr Asn Gly His Val Val
    290                 295                 300

His Asp Ala Gln His Pro Ser Arg Ser Asn Val Ala Phe Lys Lys Glu
305                 310                 315                 320

Leu His Thr Ser Gln Ser Asp Arg Ser Cys Thr His Leu Glu Phe Asp
                325                 330                 335

Ile Ser His Thr Gly Leu Ser Tyr Glu Thr Gly Asp His Val Gly Val
            340                 345                 350

Tyr Ser Glu Asn Leu Ser Glu Val Val Asp Glu Ala Leu Lys Leu Leu
        355                 360                 365

Gly Leu Ser Pro Asp Thr Tyr Phe Ser Val His Ala Asp Lys Glu Asp
    370                 375                 380

Gly Thr Pro Ile Gly Gly Ala Ser Leu Pro Pro Pro Phe Pro Pro Cys
385                 390                 395                 400

Thr Leu Arg Asp Ala Leu Thr Arg Tyr Ala Asp Val Leu Ser Ser Pro
                405                 410                 415

Lys Lys Val Ala Leu Leu Ala Leu Ala Ala His Ala Ser Asp Pro Ser
            420                 425                 430

Glu Ala Asp Arg Leu Lys Phe Leu Ala Ser Pro Ala Gly Lys Asp Glu
```

435 440 445

Tyr Ala Gln Trp Ile Val Ala Asn Gln Arg Ser Leu Leu Glu Val Met
450 455 460

Gln Ser Phe Pro Ser Ala Lys Pro Pro Leu Gly Val Phe Phe Ala Ala
465 470 475 480

Val Ala Pro Arg Leu Gln Pro Arg Tyr Tyr Ser Ile Ser Ser Ser Pro
485 490 495

Lys Met Ser Pro Asn Arg Ile His Val Thr Cys Ala Leu Val Tyr Glu
500 505 510

Thr Thr Pro Ala Gly Arg Ile His Arg Gly Leu Cys Ser Thr Trp Met
515 520 525

Lys Asn Ala Val Pro Leu Thr Glu Ser Pro Asp Cys Ser Gln Ala Ser
530 535 540

Ile Phe Val Arg Thr Ser Asn Phe Arg Leu Pro Val Asp Pro Lys Val
545 550 555 560

Pro Val Ile Met Ile Gly Pro Gly Thr Gly Leu Ala Pro Phe Arg Gly
565 570 575

Phe Leu Gln Glu Arg Leu Ala Leu Lys Glu Ser Gly Thr Glu Leu Gly
580 585 590

Ser Ser Ile Phe Phe Phe Gly Cys Arg Asn Arg Lys Val Asp Phe Ile
595 600 605

Tyr Glu Asp Glu Leu Asn Asn Phe Val Glu Thr Gly Ala Leu Ser Glu
610 615 620

Leu Ile Val Ala Phe Ser Arg Glu Gly Thr Ala Lys Glu Tyr Val Gln
625 630 635 640

His Lys Met Ser Gln Lys Ala Ser Asp Ile Trp Lys Leu Leu Ser Glu
645 650 655

Gly Ala Tyr Leu Tyr Val Cys Gly Asp Ala Lys Gly Met Ala Lys Asp
660 665 670

Val His Arg Thr Leu His Thr Ile Val Gln Glu Gln Gly Ser Leu Asp
675 680 685

```
Ser Ser Lys Ala Glu Leu Tyr Val Lys Asn Leu Gln Met Ser Gly Arg
    690                 695                 700

Tyr Leu Arg Asp Val Trp
705                 710
```

<210> 20
<211> 511
<212> PRT
<213> Rubus suavissimus

<400> 20

```
Met Ala Thr Leu Leu Glu His Phe Gln Ala Met Pro Phe Ala Ile Pro
1               5                   10                  15

Ile Ala Leu Ala Ala Leu Ser Trp Leu Phe Leu Phe Tyr Ile Lys Val
            20                  25                  30

Ser Phe Phe Ser Asn Lys Ser Ala Gln Ala Lys Leu Pro Pro Val Pro
        35                  40                  45

Val Val Pro Gly Leu Pro Val Ile Gly Asn Leu Leu Gln Leu Lys Glu
    50                  55                  60

Lys Lys Pro Tyr Gln Thr Phe Thr Arg Trp Ala Glu Glu Tyr Gly Pro
65                  70                  75                  80

Ile Tyr Ser Ile Arg Thr Gly Ala Ser Thr Met Val Val Leu Asn Thr
                85                  90                  95

Thr Gln Val Ala Lys Glu Ala Met Val Thr Arg Tyr Leu Ser Ile Ser
            100                 105                 110

Thr Arg Lys Leu Ser Asn Ala Leu Lys Ile Leu Thr Ala Asp Lys Cys
        115                 120                 125

Met Val Ala Ile Ser Asp Tyr Asn Asp Phe His Lys Met Ile Lys Arg
    130                 135                 140

Tyr Ile Leu Ser Asn Val Leu Gly Pro Ser Ala Gln Lys Arg His Arg
145                 150                 155                 160

Ser Asn Arg Asp Thr Leu Arg Ala Asn Val Cys Ser Arg Leu His Ser
                165                 170                 175

Gln Val Lys Asn Ser Pro Arg Glu Ala Val Asn Phe Arg Arg Val Phe
            180                 185                 190
```

```
Glu Trp Glu Leu Phe Gly Ile Ala Leu Lys Gln Ala Phe Gly Lys Asp
        195                 200                 205

Ile Glu Lys Pro Ile Tyr Val Glu Glu Leu Gly Thr Thr Leu Ser Arg
    210                 215                 220

Asp Glu Ile Phe Lys Val Leu Val Leu Asp Ile Met Glu Gly Ala Ile
225                 230                 235                 240

Glu Val Asp Trp Arg Asp Phe Phe Pro Tyr Leu Arg Trp Ile Pro Asn
                245                 250                 255

Thr Arg Met Glu Thr Lys Ile Gln Arg Leu Tyr Phe Arg Arg Lys Ala
            260                 265                 270

Val Met Thr Ala Leu Ile Asn Glu Gln Lys Lys Arg Ile Ala Ser Gly
        275                 280                 285

Glu Glu Ile Asn Cys Tyr Ile Asp Phe Leu Leu Lys Glu Gly Lys Thr
    290                 295                 300

Leu Thr Met Asp Gln Ile Ser Met Leu Leu Trp Glu Thr Val Ile Glu
305                 310                 315                 320

Thr Ala Asp Thr Thr Met Val Thr Thr Glu Trp Ala Met Tyr Glu Val
                325                 330                 335

Ala Lys Asp Ser Lys Arg Gln Asp Arg Leu Tyr Gln Glu Ile Gln Lys
            340                 345                 350

Val Cys Gly Ser Glu Met Val Thr Glu Glu Tyr Leu Ser Gln Leu Pro
        355                 360                 365

Tyr Leu Asn Ala Val Phe His Glu Thr Leu Arg Lys His Ser Pro Ala
    370                 375                 380

Ala Leu Val Pro Leu Arg Tyr Ala His Glu Asp Thr Gln Leu Gly Gly
385                 390                 395                 400

Tyr Tyr Ile Pro Ala Gly Thr Glu Ile Ala Ile Asn Ile Tyr Gly Cys
                405                 410                 415

Asn Met Asp Lys His Gln Trp Glu Ser Pro Glu Glu Trp Lys Pro Glu
            420                 425                 430

Arg Phe Leu Asp Pro Lys Phe Asp Pro Met Asp Leu Tyr Lys Thr Met
        435                 440                 445
```

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Ala Phe Gly Ala Gly Lys Arg Val Cys Ala Gly Ser Leu Gln Ala Met
    450                 455                 460

Leu Ile Ala Cys Pro Thr Ile Gly Arg Leu Val Gln Glu Phe Glu Trp
465                 470                 475                 480

Lys Leu Arg Asp Gly Glu Glu Glu Asn Val Asp Thr Val Gly Leu Thr
                485                 490                 495

Thr His Lys Arg Tyr Pro Met His Ala Ile Leu Lys Pro Arg Ser
            500                 505                 510
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Ser Ile Val Val Arg Trp Ala Trp Ser Val Val Asn Trp Val Trp Phe
            20                  25                  30

Lys Pro Lys Lys Leu Glu Arg Phe Leu Arg Glu Gln Gly Leu Lys Gly
        35                  40                  45

Asn Ser Tyr Arg Phe Leu Tyr Gly Asp Met Lys Glu Asn Ser Ile Leu
    50                  55                  60

Leu Lys Gln Ala Arg Ser Lys Pro Met Asn Leu Ser Thr Ser His Asp
65                  70                  75                  80

Ile Ala Pro Gln Val Thr Pro Phe Val Asp Gln Thr Val Lys Ala Tyr
                85                  90                  95

Gly Lys Asn Ser Phe Asn Trp Val Gly Pro Ile Pro Arg Val Asn Ile
            100                 105                 110

Met Asn Pro Glu Asp Leu Lys Asp Val Leu Thr Lys Asn Val Asp Phe
        115                 120                 125

Val Lys Pro Ile Ser Asn Pro Leu Ile Lys Leu Leu Ala Thr Gly Ile
    130                 135                 140

Ala Ile Tyr Glu Gly Glu Lys Trp Thr Lys His Arg Arg Ile Ile Asn
145                 150                 155                 160
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Pro Thr Phe His Ser Glu Arg Leu Lys Arg Met Leu Pro Ser Phe His
                165                 170                 175

Gln Ser Cys Asn Glu Met Val Lys Glu Trp Glu Ser Leu Val Ser Lys
            180                 185                 190

Glu Gly Ser Ser Cys Glu Leu Asp Val Trp Pro Phe Leu Glu Asn Met
        195                 200                 205

Ser Ala Asp Val Ile Ser Arg Thr Ala Phe Gly Thr Ser Tyr Lys Lys
    210                 215                 220

Gly Gln Lys Ile Phe Glu Leu Leu Arg Glu Gln Val Ile Tyr Val Thr
225                 230                 235                 240

Lys Gly Phe Gln Ser Phe Tyr Ile Pro Gly Trp Arg Phe Leu Pro Thr
                245                 250                 255

Lys Met Asn Lys Arg Met Asn Glu Ile Asn Glu Glu Ile Lys Gly Leu
            260                 265                 270

Ile Arg Gly Ile Ile Ile Asp Arg Glu Gln Ile Ile Lys Ala Gly Glu
        275                 280                 285

Glu Thr Asn Asp Asp Leu Leu Gly Ala Leu Met Glu Ser Asn Leu Lys
    290                 295                 300

Asp Ile Arg Glu His Gly Lys Asn Asn Lys Asn Val Gly Met Ser Ile
305                 310                 315                 320

Glu Asp Val Ile Gln Glu Cys Lys Leu Phe Tyr Phe Ala Gly Gln Glu
                325                 330                 335

Thr Thr Ser Val Leu Leu Ala Trp Thr Met Val Leu Leu Gly Gln Asn
            340                 345                 350

Gln Asn Trp Gln Asp Arg Ala Arg Gln Glu Val Leu Gln Val Phe Gly
        355                 360                 365

Ser Ser Lys Pro Asp Phe Asp Gly Leu Ala His Leu Lys Val Val Thr
    370                 375                 380

Met Ile Leu Leu Glu Val Leu Arg Leu Tyr Pro Pro Val Ile Glu Leu
385                 390                 395                 400

Ile Arg Thr Ile His Lys Lys Thr Gln Leu Gly Lys Leu Ser Leu Pro
                405                 410                 415
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Glu Gly Val Glu Val Arg Leu Pro Thr Leu Leu Ile His His Asp Lys
            420                 425                 430

Glu Leu Trp Gly Asp Asp Ala Asn Gln Phe Asn Pro Glu Arg Phe Ser
        435                 440                 445

Glu Gly Val Ser Lys Ala Thr Lys Asn Arg Leu Ser Phe Phe Pro Phe
    450                 455                 460

Gly Ala Gly Pro Arg Ile Cys Ile Gly Gln Asn Phe Ser Met Met Glu
465                 470                 475                 480

Ala Lys Leu Ala Leu Ala Leu Ile Leu Gln His Phe Thr Phe Glu Leu
                485                 490                 495

Ser Pro Ser His Ala His Ala Pro Ser His Arg Ile Thr Leu Gln Pro
            500                 505                 510

Gln Tyr Gly Val Arg Ile Ile Leu His Arg Arg
        515                 520
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<212> PRT
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Met Asp Pro Ser Asn Ser Ser Phe Glu Ser Thr Gly Glu Val Ala Ser
            20                  25                  30

Val Ile Phe Glu Asn Arg Glu Leu Val Ala Ile Leu Thr Thr Ser Ile
        35                  40                  45

Ala Val Met Ile Gly Cys Phe Val Val Leu Met Trp Arg Arg Ala Gly
    50                  55                  60

Ser Arg Lys Val Lys Asn Val Glu Leu Pro Lys Pro Leu Ile Val His
65                  70                  75                  80

Glu Pro Glu Pro Glu Val Glu Asp Gly Lys Lys Lys Val Ser Ile Phe
                85                  90                  95

Phe Gly Thr Gln Thr Gly Thr Ala Glu Gly Phe Ala Lys Ala Leu Ala
            100                 105                 110
```

```
Asp Glu Ala Lys Ala Arg Tyr Glu Lys Ala Thr Phe Arg Val Val Asp
        115                 120                 125

Leu Asp Asp Tyr Ala Ala Asp Asp Asp Gln Tyr Glu Glu Lys Leu Lys
    130                 135                 140

Asn Glu Ser Phe Ala Val Phe Leu Leu Ala Thr Tyr Gly Asp Gly Glu
145                 150                 155                 160

Pro Thr Asp Asn Ala Ala Arg Phe Tyr Lys Trp Phe Ala Glu Gly Lys
                165                 170                 175

Glu Arg Gly Glu Trp Leu Gln Asn Leu His Tyr Ala Val Phe Gly Leu
            180                 185                 190

Gly Asn Arg Gln Tyr Glu His Phe Asn Lys Ile Ala Lys Val Ala Asp
        195                 200                 205

Glu Leu Leu Glu Ala Gln Gly Gly Asn Arg Leu Val Lys Val Gly Leu
    210                 215                 220

Gly Asp Asp Asp Gln Cys Ile Glu Asp Asp Phe Ser Ala Trp Arg Glu
225                 230                 235                 240

Ser Leu Trp Pro Glu Leu Asp Met Leu Leu Arg Asp Glu Asp Asp Ala
                245                 250                 255

Thr Thr Val Thr Thr Pro Tyr Thr Ala Ala Val Leu Glu Tyr Arg Val
            260                 265                 270

Val Phe His Asp Ser Ala Asp Val Ala Ala Glu Asp Lys Ser Trp Ile
        275                 280                 285

Asn Ala Asn Gly His Ala Val His Asp Ala Gln His Pro Phe Arg Ser
    290                 295                 300

Asn Val Val Val Arg Lys Glu Leu His Thr Ser Ala Ser Asp Arg Ser
305                 310                 315                 320

Cys Ser His Leu Glu Phe Asn Ile Ser Gly Ser Ala Leu Asn Tyr Glu
                325                 330                 335

Thr Gly Asp His Val Gly Val Tyr Cys Glu Asn Leu Thr Glu Thr Val
            340                 345                 350

Asp Glu Ala Leu Asn Leu Leu Gly Leu Ser Pro Glu Thr Tyr Phe Ser
```

```
        355                 360                 365

Ile Tyr Thr Asp Asn Glu Asp Gly Thr Pro Leu Gly Gly Ser Ser Leu
    370                 375                 380

Pro Pro Pro Phe Pro Ser Cys Thr Leu Arg Thr Ala Leu Thr Arg Tyr
385                 390                 395                 400

Ala Asp Leu Leu Asn Ser Pro Lys Lys Ser Ala Leu Leu Ala Leu Ala
                405                 410                 415

Ala His Ala Ser Asn Pro Val Glu Ala Asp Arg Leu Arg Tyr Leu Ala
            420                 425                 430

Ser Pro Ala Gly Lys Asp Glu Tyr Ala Gln Ser Val Ile Gly Ser Gln
        435                 440                 445

Lys Ser Leu Leu Glu Val Met Ala Glu Phe Pro Ser Ala Lys Pro Pro
    450                 455                 460

Leu Gly Val Phe Phe Ala Ala Val Ala Pro Arg Leu Gln Pro Arg Phe
465                 470                 475                 480

Tyr Ser Ile Ser Ser Ser Pro Arg Met Ala Pro Ser Arg Ile His Val
                485                 490                 495

Thr Cys Ala Leu Val Tyr Asp Lys Met Pro Thr Gly Arg Ile His Lys
            500                 505                 510

Gly Val Cys Ser Thr Trp Met Lys Asn Ser Val Pro Met Glu Lys Ser
        515                 520                 525

His Glu Cys Ser Trp Ala Pro Ile Phe Val Arg Gln Ser Asn Phe Lys
    530                 535                 540

Leu Pro Ala Glu Ser Lys Val Pro Ile Ile Met Val Gly Pro Gly Thr
545                 550                 555                 560

Gly Leu Ala Pro Phe Arg Gly Phe Leu Gln Glu Arg Leu Ala Leu Lys
                565                 570                 575

Glu Ser Gly Val Glu Leu Gly Pro Ser Ile Leu Phe Phe Gly Cys Arg
            580                 585                 590

Asn Arg Arg Met Asp Tyr Ile Tyr Glu Asp Glu Leu Asn Asn Phe Val
        595                 600                 605
```

```
Glu Thr Gly Ala Leu Ser Glu Leu Val Ile Ala Phe Ser Arg Glu Gly
    610                 615                 620

Pro Thr Lys Glu Tyr Val Gln His Lys Met Ala Glu Lys Ala Ser Asp
625                 630                 635                 640

Ile Trp Asn Leu Ile Ser Glu Gly Ala Tyr Leu Tyr Val Cys Gly Asp
                645                 650                 655

Ala Lys Gly Met Ala Lys Asp Val His Arg Thr Leu His Thr Ile Met
            660                 665                 670

Gln Glu Gln Gly Ser Leu Asp Ser Ser Lys Ala Glu Ser Met Val Lys
        675                 680                 685

Asn Leu Gln Met Asn Gly Arg Tyr Leu Arg Asp Val Trp
    690                 695                 700
```

Claims

1. A method of releasing steviol glycosides from a yeast host cell, comprising the steps of:

   a) providing a fermentation medium comprising a yeast host cell engineered to produce one or more steviol glycosides; and
   b) releasing the one or more steviol glycosides from the engineered yeast host cell by heating the fermentation medium to a temperature in the range from 70° C to 75° C for 5 minutes to 48 hours, wherein the one or more steviol glycosides are released from an intracellular portion of the yeast host cell into an extracellular portion of the fermentation medium;
   wherein the steviol glycoside is selected from rebaudioside M, rebaudioside D, rebaudioside A, rebaudioside B or combinations thereof.

2. The method of claim 1, wherein the heating is 75°C for 5 minutes to 2 hours.

3. The method of claim 1 or 2, wherein the heating does not increase extracellular nitrogen content to more than 45% of the total nitrogen components of the fermentation medium.

4. The method of any one of claims 1-3, wherein the heating is for 5 minutes to 2 hours.

5. The method of any one of the preceding claims, wherein the engineered yeast host cell is selected from the group consisting of genus, *Candida, Pichia* (*Hansenula*), *Kloeckera* (*Hanseniaspora*), *Kluyveromyces, Rhodotorula, Torulopsis, Zygosaccharomyces, Saccharomycete, Yarrowia,* and *Saccharomyces.*

6. The method of any one of the preceding claims, wherein the rebaudioside M, rebaudioside D, rebaudioside A, rebaudioside B or combinations thereof released are greater than 90% of the rebaudioside M, rebaudioside D, rebaudioside A, rebaudioside B or combinations compared to the total glycosides produced and found both intracellularly and extracellularly.

7. The method of any one of the preceding claims, wherein the rebaudioside D and/or rebaudioside M released extracellularly is greater than 33 % of the total of both intracellular and extracellular rebaudioside D and/or rebaudioside M present in the fermentation medium.

8. The method of any one of the preceding claims, wherein rebaudioside D released, is present extracellularly in the range of 30% to 100% of the total amount of rebaudioside D present in the fermentation medium.

9. The method of any one of the preceding claims, wherein the rebaudioside M released, is present extracellularly in the range of 30% to 100%, of the total amount of rebaudioside M present in the fermentation medium.

10. The method of any one of the preceding claims, wherein the rebaudioside A amount, is released extracellularly in the range of 30% to 100 %, 50 % to 100 %, 80 % to 90 % of the total amount of rebaudioside A produced during the fermentation.

11. The method of any one of the preceding claims, wherein the rebaudioside B amount, is released extracellularly in the range of 25% to 100%, 30% to 95%, 40% to 80% of the total amount of rebaudioside B produced during the fermentation.

Patentansprüche

1. Verfahren zum Freisetzen von Steviolglycosiden aus einer Hefewirtszelle, das folgende Schritte umfasst:

   a) Bereitstellen eines Fermentationsmediums, das eine Hefewirtszelle umfasst, die verändert wird, um ein oder mehrere Steviolglycoside zu erzeugen; und
   b) Freisetzen des einen oder der mehreren Steviolglycoside aus der veränderten Hefewirtszelle durch Erwärmen des Fermentationsmediums auf eine Temperatur in dem Bereich von 70 °C bis 75 °C für 5 Minuten bis 48 Stunden lang, wobei das eine oder die mehreren Steviolglycoside aus einem intrazellulären Abschnitt der Hefewirtszelle in einem extrazellulären Abschnitt des Fermentationsmediums freigesetzt werden;
   wobei das Steviolglycosid aus Rebaudiosid M, Rebaudiosid D, Rebaudiosid A, Rebaudiosid B oder Kombinationen davon ausgewählt ist.

2. Verfahren nach Anspruch 1, wobei das Erwärmen 75 °C für 5 Minuten bis 2 Stunden lang beträgt.

3. Verfahren nach Anspruch 1 oder 2, wobei das Erwärmen ein Gehalt an extrazellulärem Stickstoff nicht auf mehr als 45 % der Gesamtsumme an Stickstoffkomponenten des Fermentationsmediums erhöht.

4. Verfahren nach einem der Ansprüche 1-3, wobei das Erwärmen für 5 Minuten bis 2 Stunden lang beträgt.

5. Verfahren nach einem der vorhergehenden Ansprüche, wobei die veränderte Hefewirtszelle aus der Gruppe ausgewählt ist, die aus der Gattung *Candida, Pichia (Hansenula), Kloeckera (Hanseniaspora), Kluyveromyces, Rhodotorula, Torulopsis, Zygosaccharomyces, Saccharomycete, Yarrowia* und *Saccharomyces* besteht.

6. Verfahren nach einem der vorhergehenden Ansprüche, wobei das freigesetzte Rebaudiosid M, Rebaudiosid D, Rebaudiosid A, Rebaudiosid B oder Kombinationen davon über 90 % des Rebaudiosids M, Rebaudiosids D, Rebaudiosids A, Rebaudiosids B oder Kombinationen im Vergleich zu der Gesamtsumme an Glycosiden, die sowohl intrazellulär als auch extrazellulär erzeugt und gefunden werden, beträgt.

7. Verfahren nach einem der vorhergehenden Ansprüche, wobei das extrazellulär freigesetzte Rebaudiosid D und/oder Rebaudiosid M über 33 % der Gesamtsumme an sowohl intrazellulärem als auch extrazellulärem Rebaudiosid D und/oder Rebaudiosid M, die in dem Fermentationsmedium vorhanden sind, beträgt.

8. Verfahren nach einem der vorhergehenden Ansprüche, wobei freigesetztes Rebaudiosid D in dem Bereich von 30 % bis 100 % der Gesamtmenge an Rebaudiosid D, das in dem Fermentationsmedium vorhanden ist, extrazellulär vorhanden ist.

9. Verfahren nach einem der vorhergehenden Ansprüche, wobei das freigesetzte Rebaudiosid M in dem Bereich von 30 % bis 100 % der Gesamtmenge an Rebaudiosid M, das in dem Fermentationsmedium vorhanden ist, extrazellulär vorhanden ist.

10. Verfahren nach einem der vorhergehenden Ansprüche, wobei die Menge an Rebaudiosid A in dem Bereich von 30 % bis 100 %, 50 % bis 100 %, 80 % bis 90 % der Gesamtmenge an Rebaudiosid A, die während der Fermentation erzeugt wird, extrazellulär freigesetzt wird.

11. Verfahren nach einem der vorhergehenden Ansprüche, wobei die Menge an Rebaudiosid B in dem Bereich von 25

% bis 100 %, 30 % bis 95 %, 40 % bis 80 % der Gesamtmenge an Rebaudiosid B, die während der Fermentation erzeugt wird, extrazellulär freigesetzt wird.

Revendications

1. Procédé de libération de glycosides de stéviol à partir d'une cellule hôte de levure, comprenant les étapes suivantes

   a) la fourniture d'un milieu de fermentation comprenant une cellule hôte de levure modifiée pour produire un ou plusieurs glycosides de stéviol ; et
   b) la libération du ou des glycosides de stéviol à partir de la cellule hôte de levure modifiée en chauffant le milieu de fermentation à une température comprise entre 70 °C et 75 °C pendant 5 minutes à 48 heures, le ou les glycosides de stéviol étant libérés d'une partie intracellulaire de la cellule hôte de levure dans une partie extracellulaire du milieu de fermentation ;
   le glycoside de stéviol étant choisi parmi le rébaudioside M, le rébaudioside D, le rébaudioside A, le rébaudioside B ou leurs combinaisons.

2. Procédé selon la revendication 1, le chauffage étant à 75 °C pendant 5 minutes à 2 heures.

3. Procédé selon la revendication 1 ou 2, le chauffage n'augmentant pas la teneur en azote extracellulaire à plus de 45 % des composants d'azote totaux du milieu de fermentation.

4. Procédé selon l'une quelconque des revendications 1 à 3, le chauffage étant de 5 minutes à 2 heures.

5. Procédé selon l'une quelconque des revendications précédentes, la cellule hôte de levure modifiée étant choisie dans le groupe constitué par le genre, *Candida, Pichia (Hansenula), Kloeckera (Hanseniaspora), Kluyveromyces, Rhodotorula, Torulopsis, Zygosaccharomyces, Saccharomycete, Yarrowia* et *Saccharomyces.*

6. Procédé selon l'une quelconque des revendications précédentes, le rébaudioside M, le rébaudioside D, le rébaudioside A, le rébaudioside B ou leurs combinaisons libérés étant supérieurs à 90 % du rébaudioside M, du rébaudioside D, du rébaudioside A, du rébaudioside B ou des combinaisons par rapport aux glycosides totaux produits et trouvés à la fois au niveau intracellulaire et extracellulaire.

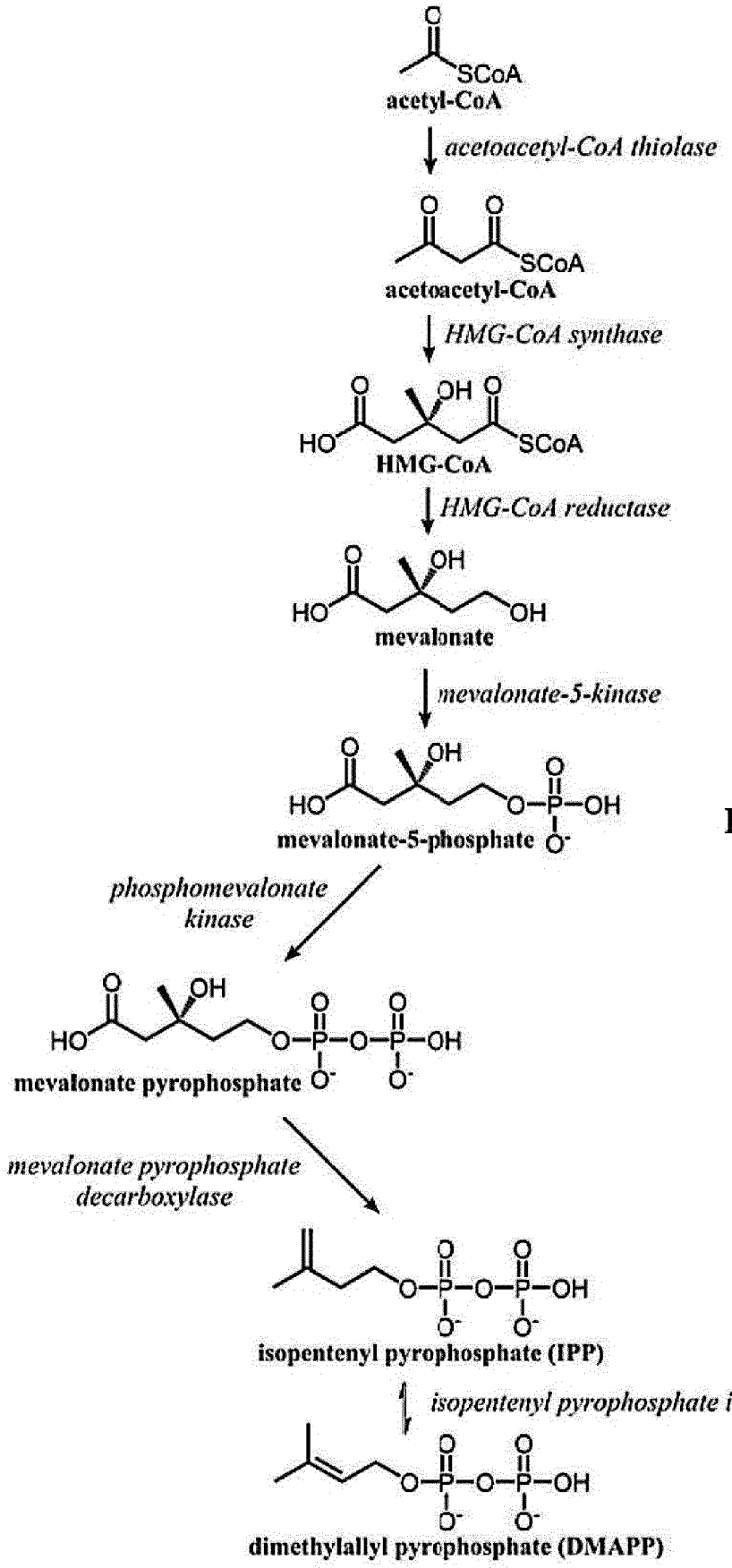
7. Procédé selon l'une quelconque des revendications précédentes, le rébaudioside D et/ou le rébaudioside M libéré de manière extracellulaire étant supérieur à 33 % du total à la fois du rébaudioside D et/ou du rébaudioside M intracellulaire et extracellulaire présents dans le milieu de fermentation.

8. Procédé selon l'une quelconque des revendications précédentes, le rébaudioside D libéré étant présent de manière extracellulaire dans la plage de 30 % à 100 % de la quantité totale de rébaudioside D présente dans le milieu de fermentation.

9. Procédé selon l'une quelconque des revendications précédentes, le rébaudioside M libéré étant présent de manière extracellulaire dans la plage de 30 % à 100 % de la quantité totale de rébaudioside M présente dans le milieu de fermentation.

10. Procédé selon l'une quelconque des revendications précédentes, la quantité de rébaudioside A étant libérée de manière extracellulaire dans la plage de 30 % à 100 %, de 50 % à 100 %, de 80 % à 90 % de la quantité totale de rébaudioside A produite pendant la fermentation.

11. Procédé selon l'une quelconque des revendications précédentes, la quantité de rébaudioside B étant libérée de manière extracellulaire dans la plage de 25 % à 100 %, de 30 % à 95 %, de 40 % à 80 % de la quantité totale de rébaudioside B produite pendant la fermentation.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acetoacetyl-CoA thiolase
acetoacetyl-CoA
HMG-CoA synthase
HMG-CoA
HMG-CoA reductase
mevalonate
mevalonate-5-kinase
mevalonate-5-phosphate
phosphomevalonate kinase
mevalonate pyrophosphate
mevalonate pyrophosphate decarboxylase
isopentenyl pyrophosphate (IPP)
isopentenyl pyrophosphate isomerase
dimethylallyl pyrophosphate (DMAPP)

FIGURE 1

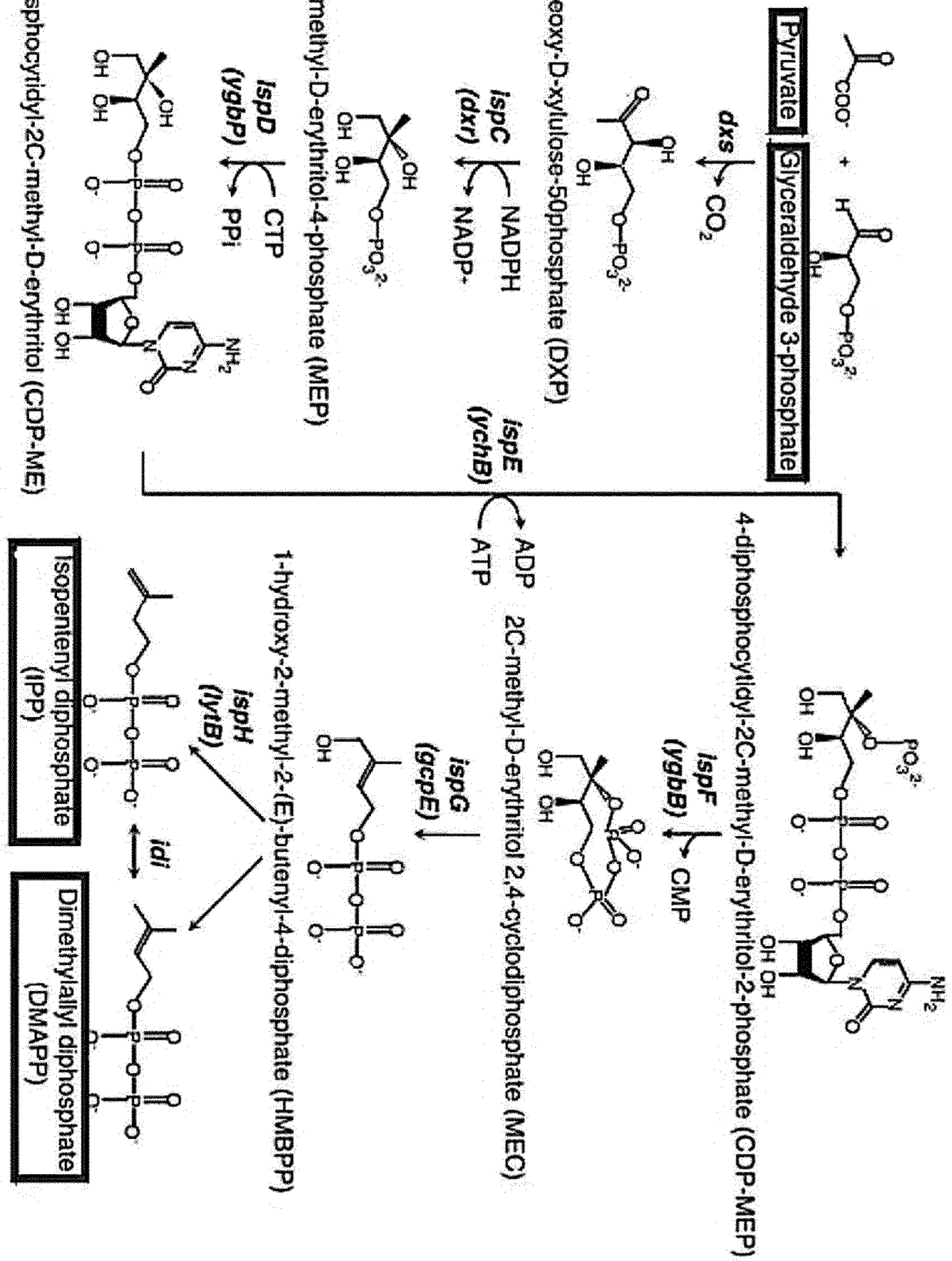

Pyruvate
Glyceraldehyde 3-phosphate
dxs
CO2
1-deoxy-D-xylulose-5phosphate (DXP)
ispC (dxr)
NADPH
NADP+
2C-methyl-D-erythritol-4-phosphate (MEP)
ispD (ygbP)
CTP
PPi
4-diphosphocytidyl-2C-methyl-D-erythritol (CDP-ME)
ispE (ychB)
ATP
ADP
4-diphosphocytidyl-2C-methyl-D-erythritol-2-phosphate (CDP-MEP)
ispF (ygbB)
CMP
2C-methyl-D-erythritol 2,4-cyclodiphosphate (MEC)
ispG (gcpE)
1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate (HMBPP)
ispH (lytB)
idi
Isopentenyl diphosphate (IPP)
Dimethylallyl diphosphate (DMAPP)

FIGURE 2

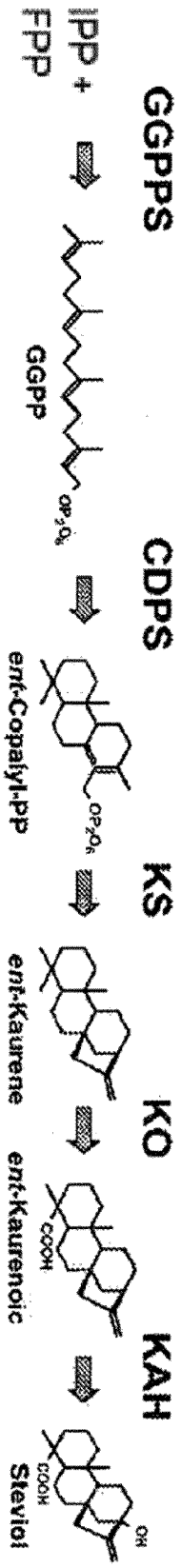
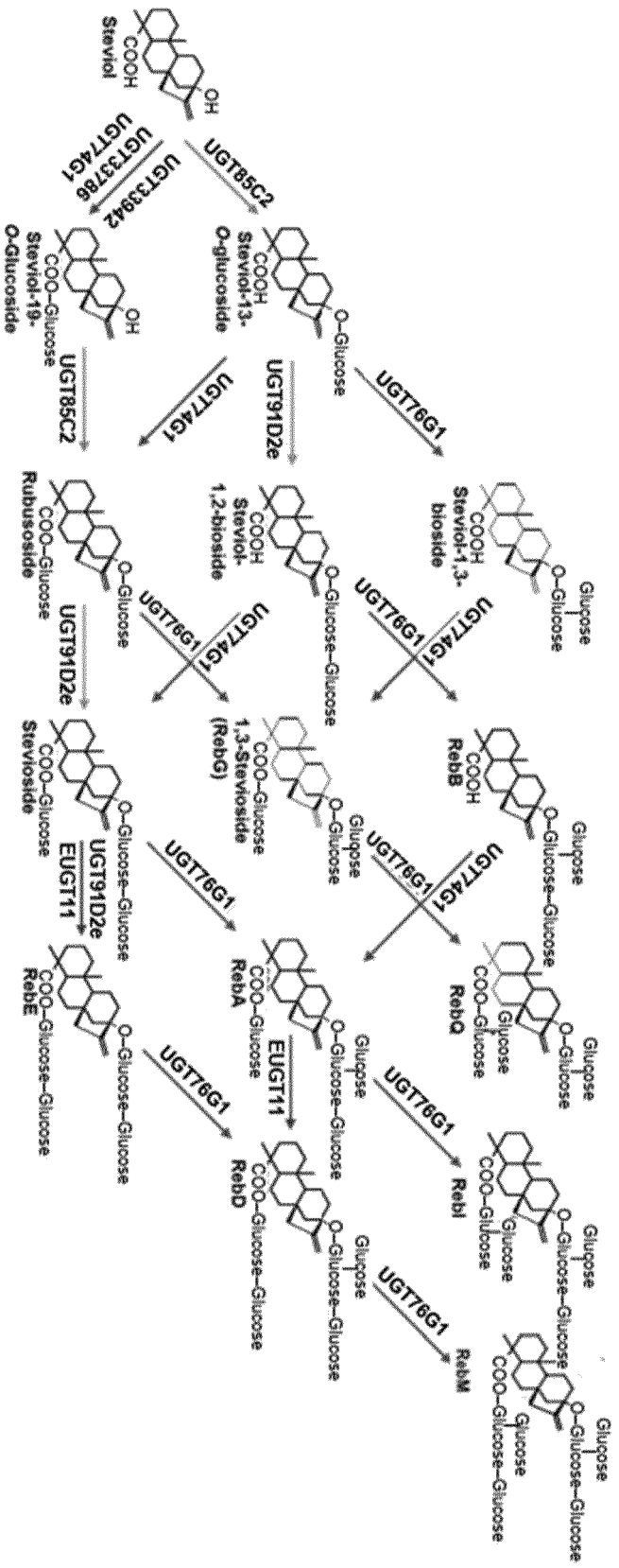

IPP +
FPP
GGPPS
GGPP
CDPS
ent-Copalyl-PP
KS
ent-Kaurene
KO
ent-Kaurenoic acid
KAH
Steviol

FIGURE 3


Steviol
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UGT74G1
UGT76G1
UGT91D2e
EUGT11
Rebl
RebM

FIGURE 4

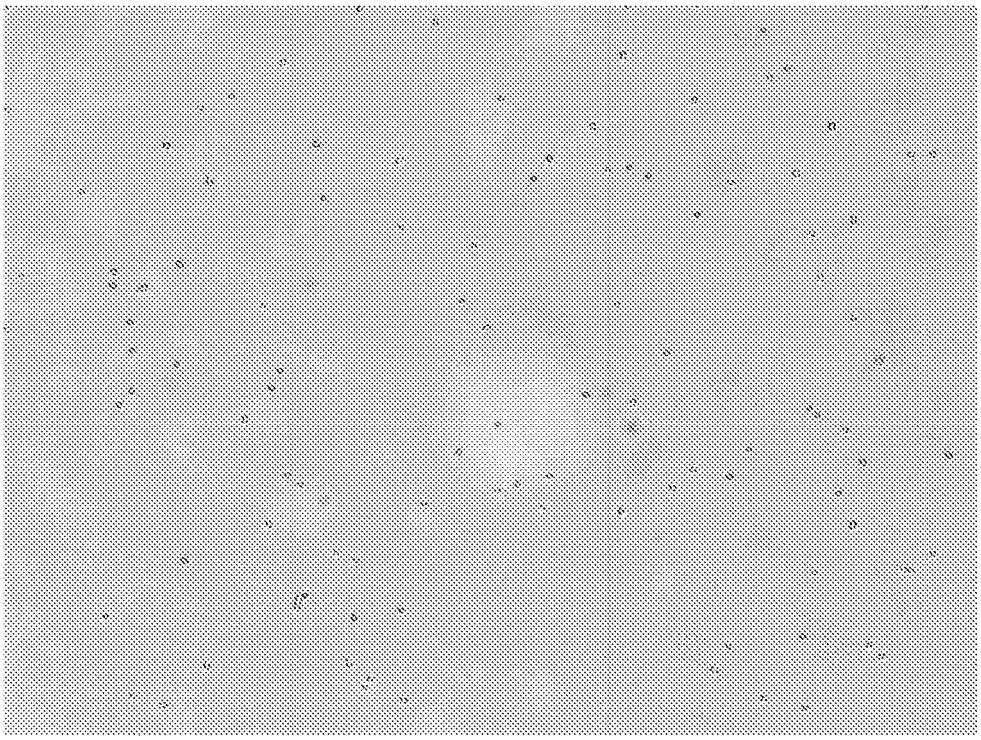

FIGURE 5 A

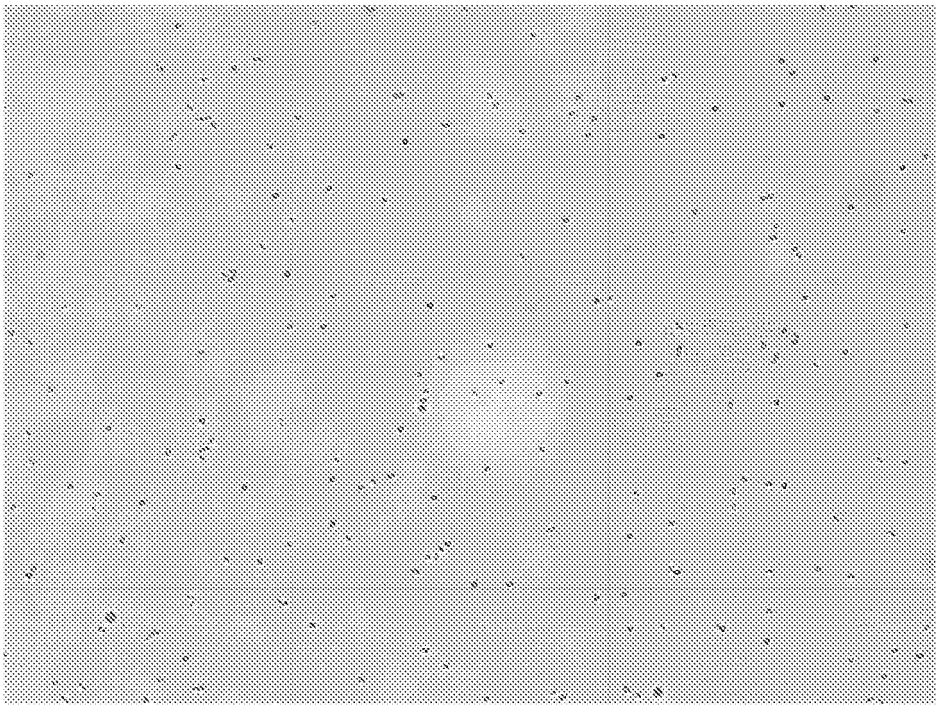

FIGURE 5 B

REFERENCES CITED IN THE DESCRIPTION

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Patent documents cited in the description

- WO 2013096420 A **[0003] [0005]**
- WO 20013096420 A **[0003]**
- WO 2013110673 A **[0007]**
- WO 2014191581 A **[0008]**
- US 2014329281 A **[0009]**
- US 20140357588 **[0044]**
- WO 2014193934 A **[0044]**
- WO 2014193888 A **[0044]**
- WO 2014122227 A **[0044] [0045] [0048]**
- WO 2011153378 A **[0048]**
- WO 2013022989 A **[0048]**
- WO 2014122328 A **[0048]**
- US 2012050021 W **[0049]**
- US 10815513 B **[0057]**
- US 2016034728 W **[0057]**
- WO 2009140394 A **[0076]**

Non-patent literature cited in the description

- **KINGHORN, A. D.** Stevia: The genus Stevia. Taylor & Francis, 2002 **[0002]**
- **OHTA.** Characterization of Novel Steviol Glycosides from Leaves of Stevia rebaudiana Morita,. *J. Appl. Glycosi.,* 2010, vol. 57, 199-209 **[0003]**
- **PRAKASH, I. et al.** *Nat. Prod. Commun.,* 2013, vol. 8, 1523-1526 **[0005]**
- **PRAKASH, I. et al.** *Int. J. Mol. Sci.,* 2012, vol. 13, 15126-15136 **[0005]**
- **LI et al.** *J. Biol. Chem.,* 2001, vol. 276, 4338-4343 **[0041]**
- **VERDUYN, C et al.** *Yeast,* 1992, vol. 8, 501-517 **[0056]**
- **VERDUYN C ; POSTMA E ; SCHEFFERS WA ; VAN DIJKEN JP.** *Yeast,* July 1992, vol. 8 (7), 501-17 **[0085]**